(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 August 2003 (07.08.2003)

PCT

(10) International Publication Number
WO 03/063793 A2

(51) International Patent Classification[7]: A61K

(21) International Application Number: PCT/US03/02954

(22) International Filing Date: 31 January 2003 (31.01.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
10/066,334 31 January 2002 (31.01.2002) US

(71) Applicant *(for all designated States except US)*: **PILOT THERAPEUTICS, INC.** [US/US]; 2000 Daniel Island Drive, Charleston, SC 29492 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **CHILTON, Floyd, H.** [US/US]; 4226 Allistar Road, Winston-Salem, NC 27104 (US). **SURETTE, Marc, E.** [CA/US]; 2344 Westover Drive, Winston-Salem, NC 27103 (US). **KOUMENIS, Iphigenia, L.** [CY/US]; 3241 York Road, Winston-Salem, NC 27103 (US). **TRAMPOSCH, Kenneth** [US/US]; 8686 Millcreek Drive, E. Amherst, NY 14051 (US).

(74) Agent: **HAGAN, Patrick, J.**; Dann, Dorfman, Herrell and Skillman, 1601 Market Street Suite 720, Philadelphia, PA 19103-2307 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/063793 A2

(54) Title: FATTY ACID-CONTAINING COMPOSITIONS AND METHODS FOR THE TREATMENT OF CYTOKINE MEDIATED DISORDERS

(57) Abstract: Compositions, dietary supplements and medical foods for the treatment of symptoms of inflammatory disorders may include gamma-linolenic acid or dihomogammalinolenic acid, an inhibitor of Δ^5 desaturase, and optionally stearidonic acid or ω-3 arachidonic acid. Preferred formulations may be in the form of a good tasting, preferably milk or fruit based drink, or a dried powder. Compositions reduce inflammation and inhibit increase in serum arachidonic acid associated with gamma-linolenic acid. The compositions of the invention may also be used for the treatment of cytokine mediated disorders in patients in need thereof.

FATTY ACID-CONTAINING COMPOSITIONS AND METHODS FOR THE TREATMENT OF CYTOKINE MEDIATED DISORDERS

By
Marc Surette
Iphigenia Koumenis
Kenneth Tramposch

Cross Reference to Related Applications

The present application claims priority to U.S. Application Number 10/066,334, filed January 31, 2002, which is a continuation-in-part of U.S. Application Number 09/644,380, filed August 23, 2000, which is the U.S. national stage of PCT/US99/03120, filed February 12, 1999, and a continuation-in-part of U.S. Serial Number 09/028,256, filed February 23, 1998, now Patent No. 6,107,334, the entire disclosures of each being incorporated by reference herein.

Field of the Invention

The present invention relates generally to the fields of lipid metabolism and dietary supplementation. More particularly, it concerns compositions and methods for controlling or reducing symptoms of inflammation or inflammatory conditions that include the use of unsaturated fatty acids, unsaturated fatty acid precursors, and/or unsaturated fatty acid analogs in nutritional formulations.

Background of the Invention

Arachidonic acid (AA) is a polyunsaturated fatty acid found in relatively small quantities in membranes of mammalian cells. Research over the last four decades has shown that the *in vivo* modulation of levels of arachidonic acid and oxygen-containing derivatives of arachidonic acid (known as eicosanoids) is intimately liked to human disease (for a review, see Samuelsson *et al.*, *Science*, 237:1171-1176, 1987 and Chilton *et al.*, In: *Crystal*, West and Barnes, eds., Lung: Scientific Foundations, Lippincott-Raven Publishers, Chapter 6, 77-88, 1997). For example, during inflammation, low levels of certain arachidonic acid derivatives render a protective response leading to enhanced disease resistance. However, these same molecules induce an autotoxic response leading to a variety of inflammatory disorders when produced in excessive quantities. Over the past three decades, the therapeutic utility of

blocking the metabolism of arachidonic acid through multiple pathways including 5-lipoxygenase and cyclooxygenase I and II has become evident for the treatment of a wide range of inflammatory disorders.

Since arachidonic acid or its precursors found in cells and tissues must be derived from diets, it follows that diet may affect diseases controlled by arachidonic acid or its derivatives. This relationship was suggested in the 1960s by studies which showed differences in frequencies of inflammatory disorders among Greenland Eskimos and Danes (Chilton *et al.*, *Biochim. Biophys. Acta*, 1299:1-15, 1996; Dyerberg and Bang, *Lancet*, ii:443435, 1979). Later studies showed similar differences between Japanese and Americans. These differences (Danes and Americans have much higher frequencies of inflammatory disorders including asthma, arthritis, psoriasis and acute myocardial infarction) were attributed, in large part, to the consumption by Danes and Americans, on Western diets, of high dietary quantities of precursor fatty acids of arachidonic acid (termed n-6 fatty acids) and arachidonic acid, offset by the low consumption of n-3 fatty acids.

Based on these observations, a number of dietary fatty acid reduction and supplementation strategies were undertaken in an attempt to influence arachidonic acid metabolism, eicosanoid production and clinical outcomes. These studies carried out over the last two decades have revealed that controlling dietary fatty acid intake in a number of animal models has great potential in reducing eicosanoid synthesis and ameliorating inflammation in models which mimic human arthritis, asthma, or glomerulonephritis (Prickett *et al.*, *J. Clin. Invest.*, 68:556-559, 1981; Kelley *et al.*, *J. Immunol.*, 134:1914-1919, 1985; Lefkowith *et al.*, *J. Immunol.*, 145:1523-1529, 1990; Rovin *et al.*, *J. Immunol.*, 145:1238-1245, 1990; Hurd *et al.*, *J. Clin. Invest.*, 67:476-482, 1981).

Leukotrienes (LT) are potent lipid mediators of inflammation that were discovered in 1979 to be the key components of the slow reacting substances of anaphylaxis (*Samuelsson, Harvey Lect.* 1979;75:1-40; *Murphy et al., Proc. Natl. Acad. Sci.*, 1979;76(9):4275-9). LT are formed by the initial conversion of AA to LTA_4 by the enzyme 5-lipoxygenase. LTA_4 is then converted to the potent inflammatory chemoattractant LTB_4 by LTA_4 hydrolase or to the peptidoleukotrienes, which include LTC4, LTD4 and LTE4, by glutathione transferase or LTC_4 synthase. LTB_4 exhibits potent chemotactic activity for neutrophils and eosinophils (*Smith et*

al., J. Pharm. Pharmoacol. 1980;32(7):517-8.) and can induce prolonged bronchial constriction, local edema formation and increased mucus in the airways (*Piper et al., Agents Actions* 1980;10(6):541-7; *Goetzl, Fed. Proc.* 1983;42(14):3128-31.). The leukotriene pathway has become a major target for the treatment of a number of inflammatory diseases including asthma. Indeed, pharmaceutical approaches for the treatment of asthma using 5-lipoxygenase inhibitors or LT receptor antagonists have proven very effective (*Kane et al., J. Allergy Clin. Immunol.* 1996;97(2):646-54; *Wenzel et al., Am. J. Respir. Crit. Care Med.* 1995;152(3):897-905.) and represent the latest therapeutic approach for the treatment of asthma.An alternative approach to controlling leukotriene biosynthesis can be achieved by the supplementation of diets with gamma-linolenic acid (GLA) which can be metabolized to compounds that inhibit leukotriene biosynthesis. Indeed, several human trials have shown that GLA effectively decreases the biosynthesis of leukotrienes by stimulated leukocytes (*Johnson et al., J. Nutr.* 1997;127(8):1435-44; *Ziboh et al., Am. J. Clin. Nutr.* 1992;55(1):39-45; *Pullman-Mooar et al., Arthritis Rheum.* 1990;33(10):1526-33). Dietary GLA is elongated in inflammatory cells to dihomogamma-linolenic acid (DGLA) and released upon cell stimulation and can either compete with AA for binding to 5-lipoxygenase or be transformed by 15-lipoxygenase to 15-hydroxyeicosatrienoic acid (15-HETrE) (*Chapkin et al., Biochim. Biophys. Acta.* 1988;959(3):322-31). The compound 15-HETrE has been shown to inhibit leukotriene formation (*Chapkin et al., Biochem. Biophys. Res. Commun.*1988:153(2):799-804; *Chilton et al., J. Immunol.* 1996;156(8):2941-7; *Ziboh et al., Am. J. Clin. Nutr.* 1992;55(1):39-45). Human neutrophils do not possess a Δ^5 desaturase activity therefore, DGLA is not converted to AA by these cells (*Chilton et al., J. Immunol.* 1996;156(8):2941-7).

In contrast to leukocytes, dietary GLA has a markedly different fate when metabolized by the liver. As in leukocytes, GLA is elongated to DGLA, however this DGLA is converted to AA by the liver Δ^5 desaturase leading to increased levels of plasma AA (*Johnson et al., J. Nutr.* 1997;127(8):1435-44; *Barham et al., J. Nutr.* 2000;130(8):1925-31). This accumulation of AA over time has the potential to negate the anti-inflammatory benefits of dietary GLA following long-term supplementation. In-vitro studies reveal that certain n-3 fatty acids, such as eicosapentaenoic acid (EPA), can prevent the desaturation of DGLA to AA possibly by inhibiting Δ^5

desaturase (*Barham et al., J. Nutr.* 2000;130(8):1925-31). Accordingly, the increase in plasma AA associated with dietary consumption of GLA was prevented in a clinical trial where diets were co-supplemented with high levels of GLA (3g/day) and EPA (3g/day) for 3 weeks (*Barham et al., J. Nutr.* 2000;130(8):1925-31).

US Patent 6,107,334 to Chilton relates to the control of inflammation using fatty acid regimens to inhibit the increase of serum arachidonic acid when GLA is provided as a dietary supplement. The entire disclosure of the '334 patent is incorporated by reference herein.

US patent 5,223,285 to DeMichele et al. discloses compositions containing high concentrations of polyunsaturated fatty acids including GLA and EPA, which are purportedly effective for the treatment of pulmonary patients suffering from acute respiratory distress syndrome (ARDS).

Public health statistics from 1980 indicate that the prevalence of asthma in the US is 4.3 %, or greater than 9.5 million individual cases. Further, prevalence of asthma is typically highest in children and the older adult population (*Pendersen, et al., Allergy, Vol. 36, pages 175-181; Wilder et al., Vital Health Stat, Vol. 10, pages 1-49, 1973*).

In view of the above, it is clear that a need exists for improved efficacious and safe compositions for the management and treatment of inflammatory disorders, such as asthma and arthritis.

Summary of the Invention

The present invention is directed to dietary strategies that treat, or reduce the side effects of lipid-mediated disorders, conditions or syndromes having an arachidonic acid metabolite component. Such components include leukotrienes, prostaglandins and lipoxins. A "lipid-mediated disorder, condition or syndrome" as used herein, refers to a condition where the over- or under-production of lipids contributes to the onset, development, evolution, severity or cause of the disorder or condition. Exemplary conditions include without limitation asthma and arthritis. Additional conditions which may be treated with the compositions of the invention are further described hereinbelow. Although GLA has been reported as beneficial in reducing symptoms of certain lipid-mediated or inflammatory conditions, unfortunately, dietary supplementation with GLA results in an increase in serum

arachidonic acid (AA), with potentially undesirable effects. In studies disclosed herein, however, it is shown that GLA supplementation does not increase AA in certain inflammatory cells. Also as disclosed herein, neutrophils, the inflammatory response cells, do not possess a Δ^5 desaturase activity, as do hepatocytes. Thus, the product of GLA elongation, DGLA cannot be converted to AA and eicosanoids in inflammatory cells. In serum, however, DGLA formed from the elongation of GLA is converted to AA via the action of a Δ^5 desaturase. This build-up of serum AA is likely to have harmful consequences in humans because increases in AA of this magnitude can increase platelet reactivity which is undesirable in most cases.

As disclosed herein, these potentially harmful effects can be circumvented by providing a Δ^5 desaturase inhibitor in combination with a leukotriene inhibitors such as GLA and/or DGLA, thus preventing the increase in serum AA levels upon GLA administration. In addition, stearidonic acid or ω-3 arachidonic acid may be provided as antagonists of arachidonic acid metabolism in immune cells, because, as shown herein, stearidonic acid is taken up by human neutrophils and elongated to ω-3 arachidonic acid, also a competitive inhibitor of arachidonic acid metabolism. It is contemplated that a buildup of ω-3 arachidonic acid in neutrophils may also result in further inhibition of the serum Δ^5 desaturation of DGLA in hepatocytes, resulting in further inhibition of serum arachidonic acid accumulation.

Described herein are compositions for diminishing symptoms of lipid-mediated disorders, conditions or syndromes. The compositions include γ-linolenic acid or dihomogammalinolenic acid, Δ^5 desaturase inhibitors, and ω-3 competitive inhibitors of arachidonic acid metabolism. In preferred embodiments the described ingredients include from around 80% to about 95% pure polyunsaturated fatty acids. Preferred Δ^5 desaturase inhibitors include eicosapentaenoic acid, sesamin, episesamin, sesaminol, sesamolin, curcumin, α-linolenic acid, heneicosapentaenoic acid, docosahexaenoic acid, alkyl gallate, propyl gallate, and *p*-isopentoxyaniline. These inhibitors may be provided as free fatty acids, fatty acyl esters, diglycerides, triglycerides, ethyl esters, phospholipids, steryl esters, sphingolipids, or a combination of these. In certain embodiments, a competitive inhibitor of arachidonic acid metabolism may be ω-3 arachidonic acid or stearidonic acid. In certain embodiments a competitive inhibitor of inflammatory cell AA metabolism and liver Δ^5 desaturase may be ω-3 AA or stearidonic acid (SA).

Preferred formulations of the disclosed compositions include flavored liquids or powders that may be rehydrated to form a drink. Preferred formulations may also include ingredients such as water, corn syrup, maltodextrin, sodium caseinate, calcium caseinate, soy protein, magnesium chloride, potassium citrate, calcium phosphate tribasic, or soy lecithin. The disclosed formulations may also include at least one emulsifying agent or emulsion stabilizer and antioxidants known in the art.

In certain embodiments, the disclosed compositions are contained in an essentially oxygen-free, air-tight container. By oxygen-free is meant the ambient air trapped within the container is essentially free of oxygen as is achieved, for example, by sealing the container in an oxidatively inert gas environment, such as a nitrogen gas environment. Preferred containers include cans or foil pouches that provide a punch-through opening for a straw. The compositions may also include a flavoring agent such as a fruit flavoring agent or a fruit juice. Other flavoring agents may include vanilla, chocolate, eggnog, berry, or other flavoring agents known in the art. Preferred antioxidants include beta-carotene, vitamin E, vitamin C, selenium, alpha tocopherol, and taurine.

Certain compositions disclosed herein may be described as milk based drinks for treatment of lipid-mediated disorders or conditions including inflammatory disorders that may include an unsaturated fatty acid portion containing γ-linolenic acid or dihomogammalinolenic acid, a Δ^5 desaturase inhibitor, and stearidonic acid or ω-3 arachidonic acid. Alternatively, certain compositions, disclosed for treatment of an inflammatory disorder, may include γ-linolenic acid or dihomogammalinolenic acid, eicosapentaenoic acid, and stearidonic acid or ω-3 arachidonic acid. Such formulations may also be used in the treatment of conditions including asthma, allergic rhinitis, allergic rhinoconjunctivitis, psoriasis, acute myocardial infarction, glomerulonephritis, Crohn's disease, inflammatory bowel disease, or arthritis, for example. The compositions are also effective for treatment of conditions that have an arachidonic acid metabolite component, such as, for example, autoimmune diseases, e.g. systemic Lupus erythematosus, schizophrenia, depression, IgA nephropathy, renal inflammation, coronary angioplasty, sepsis and toxic shock, organ failure, organ transplants, coronary angioplasty, risk reduction for Alzheimer's disease, cystic fibrosis, atherosclerosis, menstrual discomfort, cyclic breast pain, premature labor, gout, venous leg ulcers, chronic urticaria, primary dysmenorrhea, early parturition,

muscle wasting, endometriosis, and Lyme disease. Additionally, arthritis related disorders including, without limitation, ankylosing spondylitis, carpal tunnel syndrome, childhood or juvenile Arthritis, chronic back injury, fibromyalgia, gout, infectious arthritis, osteoarthritis, osteoporosis, Pagets's Disease, polymyalgia rheumatica, polymyositis and dermatomyositis, pseudogout, psoriatic arthritis, Raynaud's Phenomenon, reactive arthritis, Reiter's Syndrome, Repetitive Stress Injury, Rheumatoid Arthritis, Scleroderma and Sjögrens Syndrome may also be treated with the compositions of the invention. Furthermore, certain cancers may be treated with the compositions of the invention including, without limitation, breast cancer, colon cancer, prostate cancer, intestinal cancer, uterine cancer, ovarian cancer, squamous cell carcinoma and testicular cancer.

Arachidonic acid metabolites, as used herein, are formed by arachidonic acid metabolism and involve a series of enzymatic reactions in the 5-Lipoxygenase pathway and Cyclooxygenase pathway I. Exemplary arachidonic acid metabolites include, without limitation, leukotrienes , prostaglandins, lipoxins, 5-HPETE, 5-HETE, 5-HETE lactone, LTA_4, 5(S),6(S)-DIHETE, 5(S), 6(R)-DIHETE, LTB_4, LXA_4, LTC_4, 12(R,S), 6-trans-LTB_4, LTF_4, LTD_4, LTE_4, 20-OH-LTE_4, 20-COOH-LTE_4, 18-COOH-LTE_4, 16-COOH-LTE_3, 14-COOH-LTE_3, LTE_4-NAc, PGG_2, PGH_2, PGD_2, 13,14-Dihydra-15-keto-PGD_2, 9α,11β-PGF_2, PGJ_2, Δ12-PGJ_2, PGE_2, 9β,11α-PGF_2, PGA_2, PGB_2, 19-OH-PGE_2, 15-keto-PGE_2, 13,14-Dihydro-15-keto-PGE_2, PGE-M, $PGF_{2\alpha}$, 15-keto-$PGF_{2\alpha}$, 13,14-Dihydro-15-keto- $PGF_{2\alpha}$, PGF-M, Lipoxin A4, lipoxin B4, 15-epi-lipoxin A4, 15-epi and lipoxin A5.

The compositions disclosed herein, including milk-based liquids having an unsaturated fatty acid portion, may contain from about 80-95% pure γ-linolenic acid, eicosapentaenoic acid, and stearidonic acid. These unsaturated fatty acids may be isolated from natural sources such as plants or animal tissues, or they may be isolated from transgenic cells engineered to produce at least one of the unsaturated fatty acids. Transgenic cells are defined as cells that include at least one stable heterologous gene, that, in this case are involved in producing the desired polyunsaturated fatty acid. Such genes may encode enzymes involved in a pathway that converts a precursor into the desired product, or that produce a precursor of the desired product, for example. Transgenic cells may include animal cells, yeast cells, plant cells, bacterial cells, or

cyanobacterial cells, for example. It is also understood that such cells may be contained in an organism such as an animal, a plant, or a plant organ.

In certain embodiments, the present inventors provide a method of inhibiting increases in serum arachidonic acid in a mammal to which γ-linolenic acid (GLA) has been provided, comprising providing to the mammal a Δ^5 desaturase inhibitor. In particular aspects, the mammal has an inflammatory disorder. In particularly preferred embodiments, the Δ^5 desaturase inhibitor is eicosapentaenoic acid (EPA). Other Δ^5-desaturase inhibitors contemplated to be useful in the present invention include sesamin, episesamin, sesaminol, sesamolin, curcumin, heneicosapentaenoic acid, alkyl gallate, propyl gallate, *p*-isopentoxyaniline, and docosahexaenoic acid. In such embodiments, an ω-3 competitive inhibitor of inflammatory cell AA metabolism and liver Δ^5 desaturase activity may also be provided. Preferred examples are stearidonic acid and ω-3 arachidonic acid.

The GLA, EPA, and SA may be administered as free fatty acids or as fatty acyl esters. In particular aspects, the acyl esters may be triglycerides, ethyl esters, phospholipids, steryl esters or sphingolipids. The GLA, EPA, and SA may be administered in a single pharmaceutical or nutritional composition or as distinct pharmaceutical compositions or nutritional supplements. Preferred compositions are contained in a good tasting, milk based or juice based drink.

Particular aspects of the present invention provide a method of treating a lipid-mediated disorder, condition or syndrome in a mammal comprising providing to the mammal a γ-linolenic acid in an amount effective to increase the amount of dihomo-γ-linolenic acid (DGLA) in inflammatory cells and the circulation of the mammal; a Δ^5 desaturase inhibitor in an amount effective to inhibit the formation of arachidonic acid in the serum of the mammal; and an amount of stearidonic acid effective to inhibit arachidonic acid metabolism in immune cells; wherein the increase in DGLA in the inflammatory cells of the mammal inhibits the metabolism of arachidonic acid and decreases the inflammatory response in the mammal.

Also contemplated herein is a dietary supplement preparation consisting essentially of GLA in an amount effective to increase the DGLA level in the user, such that the DGLA inhibits the metabolism of arachidonic acid in the inflammatory cells, and an amount of a Δ^5 desaturase inhibitor, preferably, EPA, which is effective to inhibit accumulation of arachidonic acid in the serum of the user. The dietary

supplement of the invention is readily adapted for administration in unit dosage form for convenient delivery of a daily dose that consists essentially of GLA, present in an amount of from about 1 gram to about 15 grams, preferably about 1 gram to about 10 grams and most preferably about 0.5 to about 3 grams; EPA, present in an amount from about 0.1 grams to about 10 grams, preferably about 0.25 grams to about 5 grams and most preferably about 0.125 grams to about 2 grams; and, optionally, stearidonic acid (SA), present in an amount from about 0.1 gram, or even 1 gram to about 15 grams, preferably about 2 grams to about 10 grams and most preferably from about 3 grams to about 5 grams.

For the effective and safe treatment of asthma, it has been found to be essential to administer a composition which comprises GLA in the range of about 0.5 to about 3 grams of GLA and EPA in the range of about .125 to about 3 grams of EPA. In terms of mg of fatty acids per Kg body weight, the preferred ranges are about 3 to 40 mg GLA per Kg body weight and about 1 to 40 mg EPA per Kg body weight. It is particularly preferred that the range of the ratio of GLA:EPA in the composition be about 1:1 to about 6:1. Preferably, GLA is administered in an amount greater that EPA with a ratio of 1.5:1 being particularly preferred. Furthermore, the combined amounts of GLA and EPA as a percentage of the total fatty acid content of the composition should be in the range of about 10-40% by weight, preferably about 18-40% by weight, and most preferably about 30-40% by weight.

When operating below the daily dose range specified the desired effects on eicosanoid synthesis and prevention of arachidonate accumulation will not be obtained. Operating above the indicated ranges will result in the consumption of large quantities of oils and may result in undesirable effects due to the large caloric intake from these oils. Additionally, it has been determined that administration of GLA and EPA at levels which exceed the doses or in ratios other than those set forth above may result in the deleterious elevation of serum arachidonic acid levels and/or elevations of liver transaminases in the serum that may not be desirable for the chronic or long-term treatment of a disorder like asthma or arthritis. Such increases are tolerable for the short-term treatment of a patient suffering for Acute Respiratory Distress Syndrome (ARDS) who is at immediate risk of serious irreversible organ damage or death. However, chronic asthma and arthritis are chronic inflammatory diseases in which the potential deleterious effects that may result from the intake of high

concentrations or altered ratios of these polyunsaturated fatty acids must be reduced or eliminated. Finally, it was unexpectedly observed that, when operating above the specified daily dose range, the desired effects on inhibiting the synthesis of eicosanoids were not achieved, further emphasizing both the utility and necessity of delivering polyunsaturated fatty acids in the dose ranges and ratios specified above for the management and treatement of chronic asthma and potentially other chronic inflammatory disorders.

According to a preferred embodiment of the invention, a fatty acid composition consisting essentially of GLA and EPA is formulated as an emulsion for oral administration as a dosage unit that enhances bioavailability of GLA and EPA. A practical impediment to the administration of high doses of polyunsaturated fatty acids (PUFA) for counteracting leukotriene biosynthesis is the large number of gelatin capsules needed to be ingested to achieve an efficacious dose. Therefore, an emulsion (e.g., oil-in-water) in accordance with this invention which is suitable for administration to adults contains Borage oil and marine oil in amounts appropriate to deliver from about 0.5 to about 3g, and preferably, about .75g GLA and from about 0.125 to about 3g, and preferably, about .45g EPA, at least one emulsifying agent or emulsion stabilizer and water. This formulation can be conveniently administered in a once a day 10g dosage unit form. Subjects consuming this oral emulsion show enhanced bioavailability of PUFA by approximately two-fold over subjects consuming identical doses of PUFA in gelatin capsules.

The emulsion of the invention can be conveniently prepared as a pediatric formulation, and includes from about 0.2g to about 3g of GLA, preferably .225 to about .3 g of GLA, from about 0.02g to about 3g of a Δ^5 desaturase inhibitor comprising EPA, preferably .15 to about .2 g of EPA, at least one emulsifying agent or emulsion stabilizer, water and, optionally at least one ingredient selected from the group of a flavoring agent, a sweetening agent, a coloring agent or a preservative.

Methods of administration of the above-described emulsion are also provided.

Surprisingly, it has been found that relatively small doses of GLA and EPA, (i.e., less than 1g each) have demonstrable beneficial effects. For example, an adult dose of .75 g GLA and .43 g EPA alters biosynthesis of arachidonic acid metabolites, including LTB_4 in stimulated leucocytes. By contrast, administration of lesser daily amounts of GLA to adults had no appreciable effects on leukotriene levels. Similarly,

GLA in an amount from .225g to about .3g and EPA in an amount from about .15 g to about .2 g is effective for the treatment of pediatric asthma. It has been found that daily doses of GLA in excess of 6g are deleterious resulting in the elevation of certain liver enzymes. Moreover, such larger amounts have no appreciable effects on leukotriene synthesis.

In yet another aspect of the invention, the compositions and emulsions described herein may be used in the treatment of cytokine-mediated disorders. As used herein a cytokine mediated disorder is a disorder wherein cytokines, leukotrienes and prostaglandins are synthesized at aberrant levels giving rise to a variety of pathological conditions. Exemplary cytokine mediated disorders that may be treated with the compositions and emulsions of the invention include, without limitation, asthma, arthritis, allergic rhinoconjunctivitis, psoriasis, Crohn's disease, inflammatory bowel disease, autoimmune diseases, systemic Lupus erythematosus, IgA nephropathy, sepsis, toxic shock, organ failure, organ transplant, cystic fibrosis, atherosclerosis, atopic dermatitis, eczema, gout, chronic urticaria, thyroiditis, endometriosis, Lyme disease, muscle wasting, ankylosing spondylitis, carpal tunnel syndrome, childhood or juvenile arthritis, fibromyalgia, infectious arthritis, osteoarthritis, osteoporosis, Paget's Disease, polymyalgia rheumatica, polymyositis , dermatomyositis, pseudogout, psoriatic arthritis, Raynaud's Syndrome, reactive arthritis, Reiter's Syndrome, repetitive stress injury, rheumatoid arthritis, scleroderma and Sjögrens Syndrome.

Overexpression of TNF-β, TNFα, IL-1beta, Il-6, Il-5, and IL-4 are often associated with inflammatory disorders and autoimmune diseases. Accordingly agents which inhibit production of these cytokines are utilized in the methods of the invention. Such agents may be administered in a formulation suitable for inhalation. Alternatively they may be administered in microcapsule containing formulations.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that, the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

Brief Description of the Drawings

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Biochemical desaturation/elongation of essential fatty acids to polyunsaturated fatty acids.

FIG. 2. Dose-response of GLA supplementation on serum fatty acid levels.

FIG. 3. Effect of GLA supplementation (up to 12 weeks) on fatty acid levels in human serum.

FIG. 4. Dose-response of GLA and metabolites supplementation into neutrophil lipids.

FIG. 5. Effect of GLA supplementation (up to 12 weeks) on fatty acid levels in human neutrophils.

FIG. 6A and FIG. 6B. Incorporation of AA (FIG. 6A) and DGLA (FIG. 6B) into glycerolipid classes of neutrophils.

FIG. 7. Fatty acid release from stimulated neutrophils before and after supplementation.

FIG. 8. Influence of GLA supplementation on leukotriene generation.

FIG. 9. Influence of GLA supplementation on 5-lipoxygenase activity.

FIG. 10. In vitro metabolism of GLA in human neutrophils.

FIG. 11. Metabolism of ^{14}C-DGLA to products by stimulated neutrophils.

FIG. 12. Influence of 15-HETrE on leukotriene generation.

FIG. 13. In vitro metabolism of arachidonic acid, gammalinolenic acid, dihomogammalinolenic acid and eicosapentaenoic acid in human neutrophils.

FIG. 14. In vitro metabolism of stearidonic acid in human neutrophils.

FIG. 15. Metabolism of GLA by human eosinophils.

FIG. 16. Influence of borage oil on early and late asthmatic response.

FIG. 17A and FIG. 17B. The two in vivo approaches to be used in order to synthesize close structural analogues of AA without affecting circulating AA levels. FIG. 17A.

GLA supplementation in combination with EPA. FIG. 17B. Stearidonic Acid Supplementation.

FIG. 18A. Bar graph indicating inhibition of arachidonic acid synthesis in liver cells by Δ^5 desaturase inhibitor, eicosapentaenoic acid.

FIG. 18B. Percent inhibition of arachidonic acid synthesis in liver cells by Δ^5-desaturase inhibitor, eicosapentaenoic acid.

FIG. 19A. and FIG. 19B. Biosynthesis of leukotriene B_4 and its ω-oxidation products in zymosan-stimulated whole blood from subjects consuming GLA.

FIG. 20. Arachidonic acid levels in plasma isolated from healthy subjects measured at baseline and three weeks after daily supplementation with 1.5g GLA (n=5), 1.5g GLA + 0.7g n-3 fatty acids (n=10) or 1.5g GLA + 2.2g n-3 fatty acids (n=10).

FIG. 21. Comparison of GLA, DGLA and EPA content of plasma from subjects consuming GLA and EPA in gelatin capsules or in an emulsion.

FIG. 22A, FIG. 22B and FIG. 22C. Pharmacokinetics of GLA and EPA in plasma from subjects supplementing their diets with 20g of emulsified GLA and EPA.

FIG. 23. LTB_4 biosynthesis in whole blood stimulated with opsonized zymosan.

FIG. 24. Schematic diagram of the 5-Lipoxygenase Pathway.

FIG. 25. Schematic diagram of the Cyclooxygenase Pathway I.

FIG. 26. A graph showing the effects of placebo, low-dose , and high-dose emulsions containing borage oil and marine oil on TNF-alpha production. The low-dose group consumed 10g of the emulsion per day (0.75GLA, 0.5gEPA). The high-dose group consumed 15g of the emulsion per day (1.12g GLA, 0.75 EPA). The group receiving placebo consumed similar formulations for borage and marine oils were replaced with olive oil which does not contain GLA or EPA.

ABBREVIATIONS

AA, 20:4, arachidonic acid; EPA, 20:5 (n-3), eicosapentaenoic acid; LA, 18:2, linoleic acid; EFA, essential fatty acid; PUFA, polyunsaturated fatty acid; GLA, 18:3 (n-6), gammalinolenic acid; DGLA, 20:3 (n-6), dihomogammalinolenic acid; SDA, 18:4 (n-3), stearidonic acid; ω-3 AA, 20:4 (n-3); PC, phosphatidylcholine; PE phosphatidylethanolamine; PL phosphatidylinositol; GPC, sn-glycero-3-phosphocholine; GCRC, General Clinical Research Center; GC/MS, gas chromatography/mass spectrometry; NICI negative ion chemical ionization; TNF,

tumor necrosis factor; FMLP, n-formyl-methionine-leucine-phenylalanine; TLC, thin layer chromatography; HPLC high pressure liquid chromatography; LTB_4, leukotriene B_4; LTB_5, leukotriene B_5; LTC_4 leukotriene C_4; PAF, platelet activating factor; HBSS, Hank's Balanced Salt Solution; BALF, bronchoalveolar lavage fluid; EAR, early asthmatic response; LAR, late asthmatic response.

Detailed Description of the Invention

The present disclosure provides a dietary strategy, including nutritional supplements, designed to improve or at least partially alleviate symptoms of lipid-mediated disorders or conditions including inflammatory disorders by providing a combination of polyunsaturated fatty acids, preferably in a milk or juice based, good tasting drink. The compositions and methods disclosed herein arose in part from the surprising discovery that human neutrophils lack a Δ^5 desaturase activity, and that, while the use of γ-linolenic acid (GLA) in the treatment of arthritis or other inflammatory conditions leads to an increase in arachidonic acid (AA) in serum phospholipids, this increase does not occur in neutrophils. An alternate and synergistic method of inhibiting neutrophil AA metabolism and preventing serum accumulation of AA in response to increased GLA is also available in light of the present discovery. It was contemplated that stearidonic acid (18:4) would also be elongated in neutrophils to form ω-3 arachidonic acid, which would accumulate due to the lack of a Δ^5-desaturase activity (Δ^5 desaturase produces AA from ω-3 arachidonic acid). This excess of ω-3 arachidonic acid is available, then, to compete with natural AA (n-6) for enzymes (phospholipase A2 isotypes, cyclooxygenase isotypes, and 5-lipoxygenase) that convert AA to oxygenated metabolites. Concomitantly, ω-3 AA formed within the serum may be converted to eicosapentaenoic acid, possibly further inhibiting the hepatic Δ^5 desaturase, and thereby contributing to the inhibition of accumulation of serum AA.

The present disclosure, thus, represents in part, a defined, three pronged mechanism of decreasing symptoms of lipid-mediated disorders or conditions. A precursor of arachidonic acid, such as GLA, may administered to a subject in order to reduce inflammation, as in conventional treatments. GLA administration to humans has been shown to effectively block AA metabolism, block the synthesis of AA products and mitigate the clinical symptoms of inflammatory disorders. As an

additional element, the increase in arachidonic acid that is normally seen in serum fatty acids with administration of GLA may be inhibited by administering a Δ^5 desaturase inhibitor, such as eicosapentaenoic acid (EPA), for example. This combination can be utilized in humans to inhibit Δ^5 desaturation of DGLA to arachidonic acid in serum. Also disclosed herein is the synergistic step of providing for the synthesis of close structural analogs (antagonists) of AA by providing stearidonic acid, a competitive substrate of inflammatory cell elongase activity, which in this case, leads to ω-3 arachidonic acid. Thus, the antagonist of AA metabolism in the neutrophils and other inflammatory cells prevents the synthesis of the eicosanoids responsible for an inflammatory response without a concomitant increase in serum AA.

The described strategy is based on the knowledge that when GLA is administered as a dietary supplement or as a component in a medical food, an endogenous elongase activity in inflammatory cells synthesizes a close analogue of AA, DGLA (FIG. 17A). A part of the present disclosure is that certain inflammatory cells cannot further desaturate DGLA to AA because they lack a Δ^5 desaturase. However, in human circulation, GLA becomes elongated to DGLA, and then is further desaturated to AA. This leads to a marked increase in AA level in the circulation as a result of GLA administration. The increased AA in the circulation has been shown to cause potentially detrimental effects such as increased platelet reactivity in humans (Seyberth *et al.*, 1975).

The present invention includes a method of providing high concentrations of GLA to humans without causing a concomitant accumulation of serum AA. Thus, high concentrations of GLA can be administered to humans to synthesize DGLA in inflammatory cells, thereby inhibiting AA metabolism, eicosanoid synthesis and attenuating the signs and symptoms of lipid-mediated and cytokine-mediated disorders or conditions without the significant side effect of circulatory AA accumulation. Specifically in the present invention, GLA is administered to humans in combination with Δ^5 desaturase inhibitors including EPA. The present inventor has shown that this combination of GLA and the Δ^5 desaturase inhibitor, EPA, causes a marked accumulation of DGLA in the circulation and in inflammatory cell lipids without causing an increase in accumulation of AA in serum lipids. Also described herein, the n-3 fatty acid, stearidonic acid (18:4) may be elongated in neutrophils to

form ω-3 arachidonic acid (FIG. 1) resulting in a dose-dependent increase in ω-3 arachidonic acid in glycerolipids of these cells, and without an increase in the Δ^5 desaturase product of ω-3 arachidonic acid, eicosapentaenoic acid, nor an increase in AA. Thus, high levels of the AA analog, ω-3 AA, can be induced in inflammatory cells by providing inflammatory cells (in vitro or in vivo) with stearidonic acid, which may be converted to ω-3 AA to compete with natural AA (n-6) for enzymes (phospholipase A_2 isotypes, cyclooxygenase isotypes, and 5-lipoxygenase) that convert AA to oxygenated metabolites.

Thus, the present invention provides combined compositions, emulsions, dietary supplements and medical foods comprising GLA, EPA, and optionally SA, for example, for the treatment of lipid-mediated disorders or conditions such as psoriasis, rheumatoid arthritis, inflammatory bowel disease, Crohn's disease, ulcerative colitis, asthma, renal inflammation, atopic dermatitis, thyroiditis, or any other disease, syndrome, condition or disorder that is mediated by lipid inflammatory mediators. Included in the latter category are diseases such as breast cancer, colon cancer, prostate cancer, autoimmune diseases, e.g. systemic Lupus erythematosus, schizophrenia, depression, IgA nephropathy, sepsis and toxic shock, organ failure, organ transplants, coronary angioplasty, risk reduction for Alzheimer's disease, cystic fibrosis, atherosclerosis, menstrual discomfort, cyclic breast pain, premature labor, gout, venous leg ulcers, chronic urticaria, primary dysmenorrhea, endometriosis, and Lyme disease. To those skilled in the art it will be apparent that all of these conditions have an inflammatory component that includes a role for arachidonic acid metabolites.

Asthma is a bronchial hyper-responsive chronic inflammatory disorder involving a variety of cells including mast cells, T lymphocytes (specifically Th_2 cells), macrophages, granulocytes, platelets, basophils and epithelial cells (Chanarin, et al., Drugs, Vol. 47, pages 12-24, 1994; Einarsson et al., Ann NY Acad Sci, Vol. 762, pages 89-100). Asthma can be characterized both clinically and pathologically. Clinically, asthma can be defined as a recurrent disease that causes intermittent wheezing, breathlessness, and sometimes a cough with sputum production. Pathologically, asthma characteristics include airflow obstruction due to a combination of smooth-muscle contraction, mucosal edema and inflammation, and viscid mucus secretion (Kaliner et al., J. Am. Med. Assoc. 258:2851-2871, 1987).

While the disease involves both the large and small airways, the recognized pathophysiological events of asthma are a reduction in the small airway components (small bronchi and bronchioles) resulting in airway resistance, reduced forced expiratory volume and flow rates, and hyperinflation with trapping of lung air. In contrast to asthma, adult (acute) respiratory distress syndrome (ARDS) is the rapid onset of progressive malfunction of the lungs usually associated with the malfunction of other organs due to the inability to take up oxygen. The condition is associated with extensive lung inflammation and small blood vessel injury in all affected organs.

Chronic obstructive pulmonary disease (COPD) also differs clinically from asthma. COPD is a slowly progressive disease of the airways that is characterized by a gradual loss of lung function. The term COPD includes chronic bronchitis, chronic obstructive bronchitis, or emphysema or combinations of these conditions.

The fatty acid compositions that have been used for the treatment of ARDS may be undesirable for the treatment of asthmatic patients due to the high amounts of PUFAs administered which give rise to a variety of undesirable side effects, including reducing any desired inhibitory effects of PUFAs on the formation of eicosanoids such as leukotrienes, which are known to contribute to inflammation associated with asthma, and the elevation of liver transaminases.

The present invention provides methods and compositions for altering the serum arachidonic acid levels of a mammal in need of GLA supplementation by providing a Δ^5 desaturase inhibitor in an amount effective to prevent or inhibit the accumulation of AA in the serum of said mammal. In preferred aspects, the present inventor has found that EPA is an in vivo and in vitro inhibitor of Δ^5 desaturase activity in the liver of humans. Thus, administration of a combination of GLA and EPA will serve to prevent the synthesis of AA and its metabolites in neutrophils, whilst inhibiting the accumulation of AA in the serum. In addition, the present invention provides formulations of GLA and EPA in oil emulsions which demonstrate enhanced bioavailability over the ingestion of GLA and EPA in gelcapsules. Emulsifying agents and emulsion stabilizers included in the formulations of this invention are agents which stabilize the continuous and discontinuous phases of an emulsion. Emulsifying agents coat the surface of oil droplets resulting in a decrease in the surface tension and a decreased tendency for the droplets to come together and coalesce, whereas emulsion stabilizers increase the viscosity of the continuous phase

so that a greater amount of energy is required for oil droplets to move towards each other than in a less viscous solution. Suitable emulsifying agents or emulsion stabilizers for use in the present invention include, without limitation, lecithin, phospholipids, xanthan gum, guar gum, pectin, carob seed gum (locust-bean gum), tragacanth gum, methylcellulose, alginates, carrageenan, starch, modified starch, carboxymethylcellulose, gum Arabic, gelatin or the like. Additional preferred ingredients may include sucrose, glucose, aspartame, glycerol, sorbitol, sorbic acid, galactolipids, sphingolipids, cellulose, hydroxypropylmethylcellulose, malt or malt extract, casein, cholesterol, egg yolk, sodium dodecyl sulfate, benzalkonium chloride, *p*-hydroxybenzoic acid, vitamin C, vitamin E or alpha-tocopherol. A composition in the form of a dried powder may be prepared using any suitable pharmaceutical carrier(s) routinely used for preparing solid formulations. Examples of such carriers include magnesium stearate, starch, lactose, sucrose and cellulose. These methods and compositions are discussed in further detail herein below.

Sources of Fatty Acids for Use in Dietary Supplements

The fatty acyl compositions of the present invention may be obtained from a variety of sources. These acids may form part of a phospholipid, steryl ester, a sphingolipid, a glyceride, such as a di- or triglyceride or may be present as free fatty acids. For a comprehensive treatise of the synthesis of fatty acyl containing lipids, one of skill in the art is referred to "Lipid: Chemistry, Biochemistry and Nutrition" (Mead *et al., Lipid: Chemistry, Biochemistry and Nutrition*, Plenum Press, New York, 1986). More particularly, the distribution of fatty acids in tissue lipids is described in Chapter 5. Of particular relevance are chapters 11, 14, 15, 17, and 18 which describe synthesis and metabolic relevance of eicosanoids, triacylglycerols, steryl esters, phosphoglycerides and sphingolipids.

GLA may be obtained from sources such as oils of evening primrose, borage, blackcurrant, echium, various fungi and algae including Mucor, Rhizopus and Spirulina. DGLA may be synthesized from GLA or alternatively, may be obtained from a variety of animal tissues including, liver, kidneys, adrenals, or gonads. AA can also be isolated from similar tissues, or from egg yolk, and can also be found in various fungal and algal oils. EPA may be found in marine oils and various algal and fungal oils. Marine oil typically contains about 18 wt % EPA. Of course, although

rather difficult and expensive, all the fatty acids may also be chemically synthesized de novo.

The following table sets forth various representative sources of GLA and EPA and the percentage of the same in these sources.

TABLE A
Percent of Fatty Acids

		Evening Primrose Oil	Borage Oil	Black Currant Oil	E. Plantageneum Seed Oil
Fatty Acid					
Palmitic acid	16:0	5	11	6	7.1
Stearic acid	18:0	2	4	2	3.7
Oleic Acid	18:1n-9	10	18	14	15.8
Linolenic Acid	18:2n-6	74	40	48	14.3
GLA	18:3n-6	9	20	19	11.2
ALA	18:3n-3	-	<1	14	33.1
Stearidonic acid	18:4n-3	-	-	2.5-4	13.9
Other		-	7	-	0.9

The oils listed above should be administered in amounts such that the combination of GLA and EPA as a percentage of the total fatty acid content of the composition consumed is in the range of about 10-40% by weight, preferably about 18-40% by weight, and most preferably about 30-40% by weight.

It is also an aspect of the present disclosure that, because specific, purified fatty acids are desired, certain organisms may be engineered to "overproduce" these particular fatty acids, making them easier to isolate and purify. For example, bacterial cells, cyanobacterial cells, fungal cells, yeast cells, plant cells, animal cells, or even organs, organelles or whole plants or animals may be engineered to overproduce or even to secrete the fatty acids needed for the compositions disclosed herein.

For example, gene sequences may be isolated that encode a single enzyme in the pathway leading to a fatty acid product, such as a Δ^6 desaturase gene, for example, as described in U.S. Patent 5,689,050, (incorporated herein by reference), for use in the practice of the present invention, or an entire pathway may be isolated from genomic clones, as described in U.S. Patent No. 5,683,898 (incorporated herein by reference). In certain embodiments, an organism or a cell of an organism is selected that produces a precursor to a desired fatty acid, and in such cases, genes encoding the "downstream" enzyme or enzymes may be provided. It also understood that even if a

cell produces the selected fatty acid, the production may be enhanced or increased by supplying additional copies under the control of more active promoter regions, or even inducible promoters so that expression of the genes may be controlled. Such systems are well known in the art.

The present invention may be described in terms of methods of treatment and pharmaceutical compositions, but it is understood that the GLA, EPA, SA and any other fatty acid used in the practice of the present invention may be incorporated into a dietary margarine, milkshake, a fraction of whole milk, a milk product, a juice, combination of juices or fruit product or other foodstuff. Pharmaceutical and dietary compositions comprising fatty acyl components are well known to those of skill in the art and have been described in U.S. Patent Nos. 4,666,701; 4,576,758; 5,352,700; 5,328,691; 4,444,755; 4,386,072; 4,309,415; 4,888,326; 4,965,075, and 5,178,873; in European Patent Nos. EP 0 713 653, and EP 0 711 503; and in PCT Applications WO 96/31457 and WO 97/21434 (each of which is specifically incorporated herein by reference).

Δ^5 desaturase Inhibitors

As discussed earlier, AA and compounds derived therefrom are central mediators of inflammatory and allergic responses. A mechanism for ameliorating the deleterious effects of these compounds is through dietary control. One such manipulation involves the production or use of natural antagonists of AA at the sites of action of these compounds, inflammatory cells. Dietary supplementation with GLA has been shown to be effective at lowering inflammatory response, and it appears that although neutrophils (inflammatory response cells) take up GLA and elongate it to DGLA, there is no subsequent production of the eicosanoids that mediate inflammatory response. As shown herein, this effect occurs because neutrophils do not possess a Δ^5 desaturase, thus the DGLA produced is not desaturated to AA. However, although neutrophils lack a Δ^5 desaturase, other cells in the circulatory system do have Δ^5 desaturation capabilities and such cells readily elongate the supplemented GLA to DGLA and desaturate that DGLA to AA. This increased circulatory AA is a potently harmful agent, and it is this problem that is addressed as an aspect of the present disclosure. Based on the discoveries disclosed herein, this potentially harmful accumulation of AA in the circulation of GLA-supplemented

individuals can now be prevented by a concomitant provision of a Δ^5 desaturase inhibitor.

EPA is an ω-3, 20 carbon fatty acid that contains five double bonds (20:5), and as such is a structural analogue of AA (20:4). EPA has been shown to act as a Δ^5 desaturase inhibitor, presumably via a feedback inhibition mechanism. Methods of producing this fatty acid have been well described in the art (e.g. U.S. patent Nos. 5,683,898; 5,567,732; 5,401,646; 5,246,842; 5,246,841; 5,215,630 each incorporated herein by reference). The present invention, in preferred embodiments, employs EPA as a Δ^5 desaturase inhibitor to be administered in a nutritional supplement to those individuals receiving GLA supplements, in order to prevent the accumulation of AA in the circulation of said individuals.

In certain embodiments, it is contemplated that other inhibitors of Δ^5 desaturase will also be useful, such compounds include members of the sesamin family, members of the curcumin family and other fatty acids such as docosahexaenoic acid, and heneicosapentaenoic acid. U.S. Patent No. 5,674,853, which is specifically incorporated herein by reference, describes the use of lignins from the sesamin family in combination with saponin compositions as enteral formulations for treatment of infection and inflammation. Such sesamins will be useful in the context of Δ^5 desaturase inhibition as described herein.

U.S. Patent 5,336,496, incorporated herein by reference, describes other inhibitors of Δ^5 desaturase that may be useful in the context of the present invention. In general terms, the Δ^5 desaturase inhibitors described therein include lignan compounds, curcumin and piperonyl butoxide. As used herein the term "lignan" includes compounds such as sesamin, sesaminol, episesamin, episesaminol, sesamolin, 2-(3,4-methylenedioxyphenyl)- 6-(3-methoxy-4-hydroxyphenyl)-3,7-dioxabicyclo[3.3.0]octane; 2,6-bis-(3-methoxy-4-hydroxyphenyl)-3,7-dioxabicyclo[3.3.0]octane; and 2-(3,4-methylenedioxyphenyl)-6-(3-methoxy-4-hydroxyphenoxy)-3,7-dioxabicyclo[3.3.0]-octane.

Methods of producing and separating these compounds are well known to those of skill in the art. For example U.S. Patent 5,209,826 describes a method of separating sesamin and episesamin. It is contemplated that the present invention may use such methods in obtaining Δ^5 desaturase inhibitors. As such, U.S. Patent 5,209,826 is incorporated herein by reference. In other embodiments, the present

invention employs microorganisms or plants, for example, for producing fatty acids as inhibitors of Δ^5 desaturase. Such techniques are well known to those of skill in the art (e.g., Shimizu *et al.*, 1988; Shimizu *et al.*, 1989).

Methods for the synthesis of curcumin-related compounds have been described in U.S. Patent 5,679,864 (incorporated herein by reference). These methods involve reacting the enol form of a 2,4-diketone with a monocarbocyclic aldehyde in the presence of an organic amine catalyst. The reactants are dissolved in a highly polar, aprotic, organic solvent. The curcumin-related product is recovered in crystalline form by precipitation from the reaction mass and solvent recrystallization and may be further purified using chromatographic techniques. The synthesis of naturally occurring curcuminoids and related compounds is well known in the art. The skilled artisan is referred to e.g., Pedersen, *et al.*, *Ann. Chem.*, 1557-69, 1985; Arrieta *et al.*, *J Prakt. Chem.*, 334:656-700, 1991 and Roughly *et al.*, *JCS Perkins Trans I*, I, 2379-88, 1973, for guidance regarding detailed description of such synthesis and characterization.

Methods of Detection and Purification

The present invention concerns the provision, for example, as dietary supplements of a number of fatty acyl compositions. The fatty acid metabolism in circulatory and neutrophil cells has a balance of different precursors and substrates of arachidonic acid metabolism. In providing exogenous fatty acids as dietary supplementation, this baseline balance of fatty acids likely is altered. In certain instances it may be necessary to monitor the levels of the different fatty acids present in an individual's circulation and/or neutrophils. The present invention encompasses methods for the determination of the fatty acyl content of cells. These methods can also be employed for purifying fatty acids for inclusion as part of a dietary supplement. Generally, these methods will follow the methods described in the examples of the initial characterization of lipid content.

Chromatographic Methods of Detection

Briefly, one generally will isolate the lipid components of a cell as described herein. Separation of lipid components from (i) non-lipid components and (ii) each other will then permit quantitation of the different lipid species. Quantitation of

separated components may be achieved by any standard methodology, that would include photodensitometric scanning of TLC plates or scintillation counting of membrane bound or liquid samples separated by various chromatographic techniques.

Any of a wide variety of chromatographic procedures may be employed. For example, thin layer chromatography, gas chromatography, high performance liquid chromatography, paper chromatography, affinity chromatography or supercritical flow chromatography may be employed. See Freifelder, *Physical Biochemistry Applications to Biochemistry and Molecular Biology*, 2nd ed. Wm. Freeman and Co., New York, NY, 1982.

Partition chromatography is based on the theory that, if two phases are in contact with one another, and if one or both phases constitute a solute, the solute will distribute itself between the two phases. Usually, partition chromatography employs a column that is filled with a sorbent and a solvent. The solution containing the solute is layered on top of the column. The solvent is then passed through the column continuously, which permits movement of the solute through the column material. The solute can then be collected based on its movement rate. The two most common types of partition chromatography are paper chromatography and thin-layer chromatography (TLC); together these are called adsorption chromatography. In both cases, the matrix contains a bound liquid. Other examples of partition chromatography are gas-liquid and gel chromatography.

Paper chromatography is a variant of partition chromatography that is performed on cellulose columns in the form of a paper sheet. This technique may be useful in identifying and characterizing the lipid content of a particular sample. Cellulose contains a large amount of bound water even when extensively dried. Partitioning occurs between the bound water and the developing solvent. Frequently, the solvent used is water. Usually, very small volumes of the solution mixture to be separated are placed at the top of the paper and allowed to dry. Capillarity draws the solvent through the paper, dissolves the sample, and moves the components in the direction of flow. Paper chromatograms may be developed for either ascending or descending solvent flow. Two dimensional separations are permitted by changing the axis of migration 90° after the first run.

Thin layer chromatography (TLC) is very commonly used to separate lipids and, therefore, is considered a preferred embodiment of the present invention. TLC

has the advantages of paper chromatography, but allows the use of any substance that can be finely divided and formed into a uniform layer. In TLC, the stationary phase is a layer of sorbent spread uniformly over the surface of a glass or plastic plate. The plates are usually made by forming a slurry of sorbent that is poured onto the surface of the gel after creating a well by placing tape at a selected height along the perimeter of the plate. After the sorbent dries, the tape is removed and the plate is treated just as paper in paper chromatography. The sample is applied and the plate is contacted with a solvent. Once the solvent has almost reached the end of the plate, the plate is removed and dried. Spots can then be identified by fluorescence, immunologic identification, counting of radioactivity, or by spraying varying reagents onto the surface to produce a color change.

In Gas-Liquid chromatography (GLC), the mobile phase is a gas and the stationary phase is a liquid adsorbed either to the inner surface of a tube or column or to a solid support. The liquid usually is applied as a solid dissolved in a volatile solvent such as ether. The sample, which may be any sample that can be volatized, is introduced as a liquid with an inert gas, such as helium, argon or nitrogen, and then heated. This gaseous mixture passes through the tubing. The vaporized compounds continually redistribute themselves between the gaseous mobile phase and the liquid stationary phase, according to their partition coefficients.

The advantage of GLC is in the separation of small molecules. Sensitivity and speed are quite good, with speeds that approach 1000 times that of standard liquid chromatography. By using a non-destructive detector, GLC can be used preparatively to purify grams quantities of material. The principal use of GLC has been in the separation of alcohols, esters, fatty acids and amines.

High Performance Liquid Chromatography (HPLC) is characterized by a very rapid separation with extraordinary resolution of peaks. This is achieved by the use of very fine particles and high pressure to maintain an adequate flow rate. Separation can be accomplished in a matter of minutes, or at most an hour. Moreover, only a very small volume of the sample is needed because the particles are so small and close-packed that the void volume is a very small fraction of the bed volume. Also, the concentration of the sample need not be very great because the bands are so narrow that there is very little dilution of the sample.

Affinity Chromatography is a chromatographic procedure that relies on the specific affinity between a substance to be isolated and a molecule that it can specifically bind to. This is a receptor-ligand type interaction. The column material is synthesized by covalently coupling one of the binding partners to an insoluble matrix. The column material is then able to specifically adsorb the substance from the solution. Elution occurs by changing the conditions to those in which binding will not occur (alter pH, ionic strength, temperature, etc.). The matrix should be a substance that itself does not adsorb molecules to any significant extent and that has a broad range of chemical, physical and thermal stability. The ligand should be coupled in such a way as to not affect its binding properties. The ligand should also provide relatively tight binding, and it should be possible to elute the substance without destroying the sample or the ligand. One of the most common forms of affinity chromatography is immunoaffinity chromatography. The generation of antibodies that would be suitable for use in accord with the present invention is discussed below.

Pharmaceutical Compositions and Routes of Administration

The nutritional compositions of the present invention will have an effective amount of a Δ^5 desaturase inhibitor, optionally an ω-3 competitive inhibitor of AA metabolism such as stearidonic acid, and GLA, alone or in combination with other dietary supplements. Such compositions will generally be dissolved or dispersed in an acceptable carrier or medium, preferably for oral or topical administration. In certain embodiments, the compositions may be formulated for intravenous, intraarterial, intramuscular, nasal, vaginal, or anal administration, however, in certain embodiments the preferred medium is a milk-based or juice based liquid.

The phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or human, as appropriate. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredients, its use in the therapeutic compositions is contemplated. Supplementary active ingredients, such as other fatty

acid supplements, vitamins, minerals, non-steroidal anti-inflammatories, etc. can also be incorporated into the compositions.

The compounds are generally formulated for oral administration. Such pharmaceutically acceptable forms include, e.g., capsules, particularly gel capsules, or any other form currently used, including cremes, and liquids, for example syrups, suspensions or emulsions, inhalants and the like.

When formulated as emulsions, the fatty acid compositions of the invention demonstrate enhanced bioavailability over the ingestion of the same fatty acids in gel capsule form. Emulsifying agents and emulsion stabilizers included in the formulations of this invention are agents which stabilize the continuous and discontinuous phases of an emulsion. Emulsifying agents coat the surface of oil droplets resulting in a decrease in the surface tension and a decreased tendency for the droplets to come together and coalesce, whereas emulsion stabilizers increase the viscosity of the continuous phase so that a greater amount of energy is required for oil droplets to move towards each other than in a less viscous solution. Suitable emulsifying agents or emulsion stabilizers for use in the present invention include, without limitation, lecithin, phospholipids, xanthan gum, guar gum, pectin, carob seed gum (locust-bean gum), tragacanth gum, methylcellulose, alginates, carrageenan, starch, modified starch, carboxymethylcellulose, gum Arabic, gelatin or the like. Additional preferred ingredients may include sucrose, glucose, aspartame, glycerol, sorbitol, sorbic acid, galactolipids, sphingolipids, cellulose, hydroxypropylmethylcellulose, malt or malt extract, casein, cholesterol, egg yolk, sodium dodecyl sulfate, benzalkonium chloride, *p*-hydroxybenzoic acid, vitamin C, vitamin E or alpha-tocopherol.

A liquid formulation will generally consist of a dispersion of the fatty acid compositions in a suitable liquid carrier(s) for example, water and/or other solvents such as, for example, polyethylene glycols, oils, milk, phospholipids, with, in certain formulations, a suspending agent, emulsifier, preservative, anti-oxidant, flavoring, and/or coloring agents. Preferred ingredients may include any of the following: galactolipids, sphingolipids, lecithins, cellulose, malt or malt extract, gelatin, casein, cholesterol, egg yolk, sodium dodecyl sulfate, benzalkonium chloride, *p*-hydroxybenzoic acid, vitamin C, vitamin E or alpha-tocopherol. A composition in the form of a dried powder may be prepared using any suitable pharmaceutical carrier(s)

routinely used for preparing solid formulations. Examples of such carriers include magnesium stearate, starch, lactose, sucrose and cellulose.

A composition in the form of a capsule can be prepared using routine encapsulation procedures. For example, a dispersion or suspension can be prepared using any suitable pharmaceutical carrier(s), for example aqueous gums, celluloses, silicates or oils and the dispersion or suspension then filled into a soft gelatin capsule.

The composition may be administered as a single daily dose, or as split doses, up to 4 times a day if desired.

For the effective and safe treatment of asthma, it has been found to be essential to administer a composition in the form of a daily dose which comprises GLA in the range of about 0.5 to about 3 grams and EPA in the range of about .125 to about 2 grams. In terms of mg of fatty acids per Kg body weight, the preferred ranges are about 3 to 40 mg GLA per Kg body weight per day and about 1 to 40 mg EPA per Kg body weight per day. It is particularly preferred that the range of the ratio of GLA:EPA in the composition be about 1:1 to about 6:1. Preferably, GLA is administered in an amount greater that EPA with a ratio of about 1.5:1 being particularly preferred. Furthermore, the combined amounts of GLA and EPA as a percentage of the total fatty acid content of the composition should be in the range of about 10-40% by weight, preferably about 18-40% by weight, and most preferably about 30-40% by weight.

The preparation of a composition that contains the Δ^5 desaturase inhibitor (EPA), stearidonic acid, and GLA compounds alone or in combination with other supplements as active ingredients will be known to those of skill in the art in light of the present disclosure. Typically, such compositions can be prepared as liquids for capsules; solid forms or suspensions; the preparations can also be emulsified.

The dietary supplement comprising the combined Δ^5 desaturase inhibitor and GLA formulations of the present invention may be in the form of ingestible liquids. For example, European patent application number EP 0713 653 Al and EP 0711 503 A2 (incorporated herein by reference) describe fruit juices and milk based liquids that can be fortified with GLA and other dietary supplements. In alternative embodiments, the combined Δ^5 desaturase inhibitor and GLA formulations of the present invention may be incorporated into a dietary margarine or other foodstuff.

Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in liquid suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof, and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical formulations suitable for ingestion may include sesame oil, evening primrose oil, peanut oil, aqueous propylene glycol, and sterile powders. In all cases it is desirable to keep the formulation sterile and stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

The active compounds may be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts and those which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, Procaine and the like.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion, and by the use of surfactants.

The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile compositions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally,

dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient.

Upon formulation, the active ingredients will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as tablets containing measured amounts of active ingredient, with even drug release capsules and the like being employable. The amounts of active ingredients in the formulations of the present invention will be similar to fatty acid supplements currently available. Those of skill in the art are referred to the Physicians Desk Reference for more comprehensive details on currently used dosages of food supplements. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

For dietary or nutriceutical use, an inhibitor of Δ^5 desaturase, alone or in combination with other dietary supplements may be formulated into a single or separate pharmaceutically acceptable compositions. Preferably formulations include a good tasting, milk based drink, or a good tasting, juice based drink or fruit based powder. Such a drink may be contained in cans, preferably cans sealed under nitrogen or other oxidatively inert gas atmosphere. Cans may be packaged in "six packs" held together by plastic or cardboard containers for easy retail sales. The drinks may also be enclosed in individual cardboard or aluminum based or other foil containers, for example, that also provide a straw for each individual container. The drink formulations may also be provided in dried or lyophilized forms for rehydration in milk, water, juice, or other suitable solvent. In certain embodiments, a pre-measured liquid container indicating the level of liquid needed for proper rehydration may be included, and in bulk powder containers, a measuring spoon may also be provided. It is also understood that individual packets may be provided that each include enough powder for a single serving.

The invention may also be embodied in a medical food that is a specially formulated composition of essential nutrients and other special dietary requirements

to be consumed or administered under medical supervision in the treatment or management of patients displaying an inflammatory or cytokine-mediated disorder. The phrase "medical food" refers to a formulated food for use as either the exclusive or a supplemental source of nutrition for patients with limited or impaired capacity to ingest, digest, absorb, or metabolize ordinary foodstuffs or certain nutrients contained therein, or have other specific nutrient requirements where dietary management cannot be achieved by modification of the normal diet or by other foods for special dietary uses. Medical foods originated under the Orphan Drug Act (U.S. Congress, 1988), and were further defined under the Nutrition Labeling and Education Act (U.S. Congress, 1990); Paragraph (5) (A) (iv) "The term `medical food` means a food that is formulated to be consumed or administered enterally under the supervision of a physician and that is intended for the specific dietary management of a disease or condition for which distinctive nutritional requirements, based on recognized scientific principles, are established by medical evaluation."

The definition makes clear that medical foods are foods that are specifically and specially formulated and processed (as opposed to naturally occurring foodstuffs used in their natural state). They are for the partial or exclusive feeding of a patient who is seriously ill or who requires the product as a major treatment modality. Medical foods are fed by means of oral intake or by enteric feeding via tube infusion; that is, medical foods provide nutrition via the gastrointestinal tract, by mouth, or through a tube or catheter that delivers nutrients beyond the oral cavity.

Medical foods are distinguished from foods for special dietary uses or from foods that make health claims by the requirement that medical foods must be used under medical supervision. The intended use of a medical food is for the dietary management of a patient receiving active and ongoing medical supervision, and the medical food is determined by the physician as a requirement to overall medical care. The medical-food category is further defined through five sub-classifications: nutritional-complete formulas, intended to provide all nutrients necessary for sustaining life viability; modular formulas, intended as prepared diets for mitigation or management of a disease; special products for inborn errors of metabolism, for correction of metabolic deficiencies from birth; oral rehydration solutions, to correct dehydration; and very low-calorie diets (less than 400 kcal/d), a regimen for special diets.

Thus, in this embodiment, the present invention encompasses a medical food comprising an orally administered emulsion containing marine and borage oils and related nutrients intended to mitigate a medical condition, such as a cytokine-mediated disorder, (e.g., asthma). In an exemplary emodiment, the medical food of the invention comprises the aforementioned oils and at least one macronutrient selected from the group consisting of protein, carbohydrates, and, optionally, additional fats formulated for human and/or animal consumption. The medical food of the invention may optionally comprise vitamins. Like the dietary supplements discussed above, the medical foods of the invention may be formulated into drinks such as fruit juices, milk etc. The dietary supplements and medical foods of the invention may be used in any suitable form, such as solid bar, as a paste, gel, tablet, capsule or liquid.

The medical food of the invention comprising marine and borage oils is not a naturally occurring foodstuff used in its natural state. The medical food is specially formulated to contain the quantities of GLA and n-3 fatty acids required to inhibit leukotriene synthesis and prevent increases in tissue AA content. In order to deliver the required quantities of fatty acids, concentrated refined oils are utilized and are formulated into an emulsion to maximize the bioavailability of the oils and provide a convenient form by which the formulation can be consumed. It is intended for partial feeding of a patient by means of oral intake.

The medical food of the invention is intended for the dietary management of asthma patients who have a nutritional requirement for anti-inflammatory fatty acids to counter their overproduction of leukotrienes. This dietary management cannot be achieved by modification of the normal diet alone since the daily intake of GLA required for the beneficial effects cannot be achieve by consumption of normal foodstuffs due to the very low levels of GLA occurring in foods.

The medical food described herein provides dietary fatty acids which can block the overproduction of leukotrienes in asthmatics. Leukotrienes are products obligatorily derived from dietary polyunsaturated fatty acids which have been shown to play a role in the pathogenesis of asthma. The medical food therefore provides unique nutrient needs for asthmatics who overproduce leukotrienes. Although the population as a whole may benefit from the consumption of the anti-inflammatory fatty acids contained in the medical food described herein, the product is specifically

designed to decrease the elevated leukotriene production associated with asthma.

Finally, the medical food of the invention will be marketed directly to physicians as a treatment to be included in the management of asthma as they see fit.

The medical foods of the invention may be administered to a patient in an effective amount and on a suitable schedule to ameliorate the symptoms of an inflammatory or cytokine mediated disorder. The appropriate dosage and schedule of administration will be determined by the physician according to the age, weight and medical condition of the patient.

The following described materials and methods were used in the studies described in Examples 1-13, below, unless otherwise indicated.

Cell and serum preparations

Neutrophils are obtained from venous blood of healthy human donors as described (Lykens *et al., Am. J. Physiol, Lung Cell Mol. Physiol.*, 262:L169-L175, 1992).

Eosinophils are purified by negative, immunomagnetic selection using monoclonals against FcKRIII (CD 16) present on neutrophils. Antibody tagged neutrophils are then incubated with anti-mouse IgG conjugated magnetic beads and removed by filtration over a magnetized steel wool column.

Monocytes are obtained as follows: a mononuclear cell layer is obtained from normal human blood after centrifugation over isolymph and washed in HBSS without Ca^{2+} or Mg^{2+}, with 0.1% gelatin and 2 mM glucose, pH 7.4. Mononuclear cells are further separated by centrifugation over discontinuous Percoll gradients (45°/50.5%, 15 min, 300xg) to obtain a rough separation of monocytes from lymphocytes, washing, and then centrifugation over 48% Percoll (15 min, 300 x g) to remove contaminating lymphocytes.

In order to obtain Alveolar Macrophage (AM), BAL fluid samples are strained through a monolayer of coarse mesh surgical gauze and total cell counts and differentials determined. Cells are pelleted, resuspended in PBS, and washed 3 times. In normal individuals, ~1 to 1.5 x 10^7 total cells are expected with ~85% of harvested cells being AM. When necessary (to attain a population of at least 85% AM), AM are further purified by centrifugation (300 x g, 15 min) over 48% Percoll. Cells are washed (3 x in buffer and resuspended at 1 X 10^7 cells/ml in HBSS.

Serum is extracted from 2 ml of venous blood from donors. Briefly, blood samples are incubated at 37°C for 30 min. Blood clots are removed from the serum by centrifugation (600 x g, 10 min). Residual red blood cells are removed from the serum by centrifugation using a Beckman Microfuge E for 5 min. After the addition of 1.9 ml of water to a 0.1 ml aliquot of the serum, lipids are extracted by the method of Bligh and Dyer (Bligh and Dyer, *Can. J. Biochem. Physiol.*, 37:911-920, 1959); Chilton *et al.*, *J. Biol. Chem.*, 258:7268-7271, 1983). A portion (5 %) of the extracted lipids is used to determine the mole quantities of fatty acids by GC/MS. Serum components are isolated into individual glycerolipid classes by TLC (System II or normal phase HPLC (Bligh and Dyer, 1959).

Chromatography Techniques

Phospholipid classes (PE, PS, PL and PC) are separated by normal phase HPLC using an Ultrasphere-Si column (4.6 x 250 mm) eluted initially with hexane:2-propanol:ethanol:25 mM phosphate buffer (pH 7.4):acetic acid (490:367:100:30:0.6, v/v) at a flow rate of 1 ml/min. After 5 min, the composition of the phosphate buffer is increased to 5 % over a 10 min period to elute all phospholipids.

TLC of phospholipid subclasses. Phospholipid subclasses (diacyl-, alkylacyl-, alky-1-enylacyl-) are separated as diglyceride acetates or benzoates on silica gel G plates developed in benzene/hexane/ ether (50:45:4, v/v). Briefly, the phosphobase moiety of phospholipids is removed by phospholipase C hydrolysis followed by the addition of acetic anhydride/pyridine (5: 1, v/v).

Leukotrienes are separated by reverse phase HPLC utilizing an Ultrasphere ODS column (2.1 mm. x 250 mm: Rainin Instrument Co, Woburn, MA) eluted with methanol/water/phosphoric acid (550:450:0.2 v/v, pH 5.7) as the mobile phase at 0.3 ml/min. After 5 min the methanol composition of the mobile phase is increased from 55% to 100% over a 20 min period. The mole quantities of each leukotriene are determined by examining its UV optical density at 270 nm. Individual peaks are integrated and their recoveries normalized by comparing these integrated areas to that of PGB_2 added as an internal standard.

GC/MS analysis of fatty acids and lipid mediators

Free fatty acids are obtained from glycerolipids by base hydrolysis using 2 N KOH (30 min, 60°C). After the addition of an equal volume of water, the pH of the reaction mixture is adjusted to 3 using 6 N HCl. Free fatty acids are then extracted with ethyl ether and converted to pentafluorobenzylesters using an equal volume of 20% pentafluorobenzylchloride in acetonitrile and 20% diisopropylethylamine in acetonitrile. The carboxylate anion of all fatty acids of interest and [$^2H_3$]-stearic acid and [$^2H_8$]-arachidonate (internal standard) are analyzed by NICI GC/MS using a Hewlett Packard mass spectrometer (HP 5989A).

Eicosanoids from ethyl acetate extracts of supernatant fluids are converted to methoxime-pentafluorobenzyl ester trimethylsilyl derivatives. These derivatives of LTB_4, LTB_5, $^2H_4$-LTB_4, PGE_2, PGE_1, $^2H_4$ PGE_2 and $^2H_4$ PGE_1 (internal standard) are analyzed on an HP selective mass detection system (Hewlett Packard 5989A) by selected ion monitoring techniques to record carboxylate anions at m/z 479, 477, 483, 524, 526, 528 and 530, respectively.

Urinary LTE_4

Aliquots of urine are spiked with ^{3}H-LTE_4 and stored at -70°C. Urinary LTE_4 is then measured using the methods of Manning *et al.*, *J. Allergy Clin. Immun.*, 86:211-220, 1990 utilizing reverse phase HPLC followed by RIA: (Christie, *J. Lipid Res.*, 26:607-612, 1985). Recovery is determined using the added [^{3}H]-LTE_4 as an internal standard. LTE_4 levels are expressed relative to urinary creatinine.

Subjects and controlled diets

Subjects are recruited by poster advertisements from the Medical Center staff and students. Inclusion criteria require healthy, normal men and women of all races, 21 to 55 years old; subjects who consume an omnivorous, nutritionally adequate diet consisting of at least 25% of calories from fat. Volunteers who are within 10% of ideal body weight (IBW) and do not exceed 30% and 35% body fat for men and women, respectively (as determined by anthrometric measurements in the GCRC). Diet compositions are determined by the food frequency questionnaire component of the Health Habits and History Questionnaire developed by the NCI (Shin *et al.*, *Am. J. Respir. Crit. Care Med.*, 149:660-666, 1994; Wenzel *et al.*, *Am J. Respir. Crit. Care Med.*, 156:737-743, 1997).

Exclusion criteria include persons with any chronic or acute disease as determined by self report or physical screening; who are vegetarians or vegans; who are lactose or egg intolerant; who use drugs that affect AA release and subsequent metabolism (steroidal and non-steroidal anti-inflammatories); with serum cholesterol levels above 220 mg/cd; who are unable or unwilling to strictly adhere to a precise, restricted diet; who are unwilling to be randomly assigned to the diet group for whatever protocol the subject volunteers; who are smokers.

Composition of the diets are based on the USDA Handbook 8 and The Nutrition Data System from The Nutritional Coordinating Center of the University of Minnesota. For each of the protocols outlined above, the menus are designed with adjustments for each subject's energy needs. Basal energy expenditure is determined by the Harris-Benedict Equations:

Basal energy expenditure (BEE) for men = 65 + (13.7 x Wt(kg)) + (5 x Ht(cm)) - (6.8 x age(yr))

for women = 655 + (9.6 x Wt(kg)) + (1.8 x Ht(cm))- (4.7 x age(yr)).

Total daily energy needs equal the BEE times an activity factor of from 1.3 for ambulatory but sedentary to 1.5 for the more active persons. Body weight is monitored each day when the subjects come to the Center to receive their meals. Calorie levels are adjusted appropriately.

Procedures and Specimens Collection used in Human Model of Atopic Asthma

Clinical data on each patient is entered into a database consisting of the following elements. Demographic data (age, sex, race, smoking history), and the data elements used to fulfill the above diagnostic criteria, spirometric data, presence of atopy (positive "prick" skin testing to respirable antigens), presence of LAR to inhaled antigen, and presence of allergic rhinoconjunctivitis.

Allergen skin testing - Atopic asthmatic subjects are identified by skin testing using the skin prick method at a 1:10 (wt/vol) dilution of 20 stock antigen solutions (Greer Laboratories, Lenoir, NC). Subjects must not be receiving immunotherapy, nor may

they be treated with systemic corticosteroids for a minimum of 4 wk. Short acting antihistamines are avoided for at least 24 h and long acting for at least 7 days. Atopic subjects are defined as those with a positive response consisting of a wheal of at least 3 by 3 mm to one or more antigens, with an appropriately negative saline control.

Allergen Inhalation Challenge - The immediate (early) asthmatic response (EAR) or late asthmatic response (LAR) is studied under controlled conditions using inhaled antigenic challenge in volunteer patients with asthma using a previously described protocol (Smith *et al.*, *Clin. Pharm. Ther.*, 54:430-436, 1993). Atopic asthmatics undergo inhaled allergen challenge followed by BAL according to the following protocol. Subjects must have no lung disease other than asthma, and, on the day of testing, must have a baseline FEV_1 >70% of predicted. Subjects must not be receiving immunotherapy, nor may they be treated with cromolyn sodium or corticosteroids (inhaled or systemic) or leukotriene antagonist for a minimum of 4 wk. Short-acting antihistamines are avoided for at least 24 h and intermediate acting for 7 days (astemizole for 6 weeks). Theophylline preparations are withheld for 24 h prior to challenge and beta-agonists for 8 h prior to challenge. On the day of challenge, subjects must be wheeze-free, with an FEV_1 > than 80% of the previously observed highest value. If the patient has an intercurrent respiratory infection, inhalation challenge is postponed for at least 6 wk. Antigens to which the subject is perennially exposed (e.g., mite, cat) are utilized whenever possible to minimize the impact of seasonal variations in environmental exposure to the specific antigen. Further, antigen testing is conducted out of the respective allergen season, or after attempts to minimize environmental exposure (e.g., to mites or cats) have been implemented. Antigenic challenge generally begins between 7:30 and 8:00 am and the patient is monitored for a minimum of 12 h following antigenic challenge. Subjects inhale allergen to which they have previously demonstrated skin sensitivity beginning at 1: 1,000,000 dilution (wt/vol) and proceeding with logarithmically increasing concentrations to 1:100. The subject breathes quietly from a continuous hand-held nebulizer for 2 min at each concentration. Following each concentration, the FEV_1 is measured at 5 min intervals (DS Plus, Warren E. Collins, Inc., Braintree, MA). If the FEV_1 does not fall by 20% after 15 min, the next higher concentration is administered. Once a 20% drop in FEV_1 is measured, or following the highest

concentration, spirometry is performed every 15 min for the first hour and then hourly for the next 11 h. Patients experiencing symptomatic bronchospasm following initial antigenic inhalation may receive a short acting inhaled beta-agonist bronchodilator agent (isoproterenol). Thi S, has no effect on the subsequent late asthmatic response (LAR). An LAR is defined as a 15% or greater fall in FEV_1 from the prechallenge baseline value occurring between 3 to 12 h after challenge.

Bronchoalveolar lavage- Subjects whose FEV_1 immediately preceding bronchoscopy is less than 60% of prechallenge baseline do not undergo BAL to minimize further acute diminution of lung function and to maximize subject safety. Fiberoptic bronchoscopy is performed following methodologies previously detailed in the literature (Wenzel *et al.*, *J. Allergy Clin. Immunol.*, 87:540-548, 1991; Zehr *et al.*, *Chest*, 95:1059-1063, 1989). Briefly, the fiberoptic bronchoscope is introduced into the lower airways trans-nasally following nebulized 4% Xylocaine, topical anesthesia and benzodiazepine sedation, titrated to patient comfort Isoproterenol, 1 puff, 130 μg is administered 10 min before bronchoscopy. Bronchoalveolar lavage (BAL) is obtained from the right middle lobe or lingula utilizing six 50 ml aliquots (200 ml total volume) of sterile normal saline without preservatives, warmed to 37°C. The amount of BAL returned is recorded and the specimen promptly processed. The right middle lobe or lingula is routinely used to maximize the uniformity of specimen yield as the return from BAL is dependent upon many factors, but especially airway geometry and gravity. These areas tend to drain spontaneously by gravity in supine patients. This improves the return of fluid from the lavage as well as minimizing the amount of retained fluid within the lung in these patients.

BAL samples are strained through a monolayer of coarse-mesh surgical gauze and total cell yield determined by taking a small aliquot of the pooled, well mixed fluid, and counting the cells in a Neubauer hemocytometer. BAL cell count is expressed as the total number of cells recovered by lavage and as the number of cells per ml of recovered BAL fluid. A small aliquot is then cytocentrifuged (Shandon Southern Cytospin) for 5 min at 4,500 RPM, air dried, and stained by a modified Wright-Giemsa stain. A 300 cell differential count is performed where alveolar macrophages and other leukocytes are enumerated. The number of ciliated or squamous epithelial cells present are noted, but are not included in the differential

count. Quantification of the cellular populations recovered by lavage are expressed as a percentage of the total cells recovered (excluding red blood cells and epithelial cells), and as the total numbers of each cell type recovered. The remaining BAL fluid is centrifuged at 500 x g, 4°C, for 15 min. Aliquots of the supernate not immediately processed are stored at -80°C.

Effect of supplementation on eosinophil eicosanoid biosynthesis: 75 ml from a peripheral vein is collected 30 to 60 min prior to inhaled challenge and 24 h after challenge. Eosinophils are isolated as described above. Cells are challenged with A23187 (1 μM) and PAF (1 μM). Leukotrienes are quantified after reverse phase HPLC as described above. Quantities of free fatty acid and prostaglandins are determined by NICI GC/MS.

Urinary LTE_4: Urine is collected for 3 h beginning immediately after antigen challenge and again from 3 h until after the LAR. Urinary LTE_4 is measured using an RIA as described above.

Arachidonic acid release: Free fatty acid levels including AA in BAL are determined, after addition of $^2H_3$-stearidonic acid and $^2H_8$-AA to BAL as internal standards, by NICI-GC/MS.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1

In Vivo Studies Examining GLA Supplementation In Humans

Incorporation of supplemented fatty acids into serum lipids

Initial studies examined the effect of dietary supplementation with GLA on the fatty acid content of serum lipids. Here, 9 healthy adult volunteers consumed a

controlled eucaloric diet consisting of 25% fat, 55% carbohydrate and 20% protein prepared in the metabolic kitchen of the GCRC.

Four menus were served on a rotating basis throughout the study period. In addition, three groups of three volunteers supplemented this diet with three different doses of GLA.

FIG. 2 demonstrates the effect of GLA supplementation at three different doses on serum levels of GLA, DGLA, and AA. In all three groups of subjects, AA significantly increased in serum lipids at the end of the three-week dietary period when compared with baseline values. Both GLA and DGLA significantly increased in the groups receiving 3.0 g/day and 6.0 g/day. In the two highest dose groups, DGLA levels increased two-fold and AA levels increased approximately 30% when compared to baseline values of these fatty acids in the same subjects. There was no significant change in serum fatty acid levels of volunteers eating control (25% energy as fat) diets, but not receiving the GLA supplement.

An important difference between the aforementioned studies and most clinical trials in the literature was the length of time of supplementation. Therefore, a long-term supplementation study (3.0g/day) was performed over a 12-week period to assess whether fatty acid ratios and distribution would change in a manner that was not observed at three weeks. This study showed that there was a significant increase in serum GLA, DGLA, and AA levels by two weeks and that these levels stayed high over an additional 10 weeks of supplementation (FIG. 3). Taken together, these data suggest that although some dietary GLA remains in the serum unchanged, substantial quantities of the elongation product (DGLA) and the elongation/ Δ^5 desaturase product (AA), accumulate in serum after GLA supplementation.

The next set of studies was designed to determine the distribution of supplemented fatty acids or their metabolites within individual glycerolipid classes of serum. Serum was collected from volunteers before and after receiving 6.0 g/day of GLA. Serum glycerolipids were separated by TLC and fractions were analyzed for fatty acid content following base hydrolysis by NICI-GC/MS. GLA was located predominately in triglycerides (36-38% of total), phospholipids (26-33% of total), and cholesterol esters (17-21 % of total). After supplementation, GLA significantly increased in both phospholipids and cholesterol esters. In contrast, DGLA and AA were almost exclusively located in serum phospholipids, with very little of these fatty

acids found in other serum pools. After supplementation, both DGLA and AA increased only in phospholipid pools.

Incorporation of supplemented fatty acids into neutrophil lipids

The fatty acid composition of the neutrophil lipids in subjects eating a controlled diet supplemented with 1.5, 3.0, or 6.0 g/day of GLA were also analyzed. No consistently detectable amounts of GLA were found in the glycerolipids of neutrophils before or after supplementation. Although relatively large quantities of AA were found in unsupplemented neutrophils, there was no significant change in AA within glycerolipids after supplementation at any of the doses given (FIG. 4). In contrast, DGLA within glycerolipids increased as a function of the dose provided to the volunteers. The AA/DGLA ratio decreased from approximately 5.4:1 before supplementation to 2.3:1 three weeks after 6.0 g/day of GLA supplementation. There was no significant change in fatty acid levels in control subjects eating the study diet without supplementation. These findings suggest that neutrophils rapidly elongate GLA to DGLA but lack the ability to desaturate DGLA to AA.

The influence of long term (12 week) GLA supplementation (3g/day) on the composition of GLA, DGLA and AA in neutrophil lipids also was examined. In contrast to serum, GLA supplementation resulted in an increase in DGLA but not AA even at 12 weeks (FIG. 5). It is not clear why the increase of AA in serum is not eventually observed in neutrophil lipids; perhaps this AA is in a serum pool not available to neutrophils. Taken together these preliminary data indicate that GLA provided as a dietary supplement is converted to different products (DGLA in inflammatory cells and AA in serum) depending on where it is metabolized. This results in the potentially beneficial effect of reducing AA metabolism in inflammatory cells balanced against the potential adverse effects of the accumulation of serum AA levels. These studies led to studies designed to determine whether it is possible to utilize the endogenous elongase activity within inflammatory cells to synthesize analogs of AA from appropriate dietary precursors without concomitantly increasing levels of circulating AA.

To better determine the distribution of fatty acids within different glycerolipid classes, neutrophils were obtained before and after supplementation with 6.0 g/day of GLA for 3 weeks and glycerolipids were separated by normal phase HPLC.

Quantities of fatty acids in each glycerolipid class were then determined by NICI-GC/MS. As shown in FIG. 6A, the majority of AA (>60%) within the neutrophil lipids was located in phosphatidylethanolamine (PE) and neither the absolute amount nor its relative distribution changed significantly after dietary supplementation with GLA. Similarly, the bulk of DGLA in the neutrophil was associated with PE (40%) (FIG. 6B). There were significant increases in the amount of DGLA associated with both PE and neutral lipids after supplementation, For example, the AA/DGLA ratio in PE decreased from 8.3:1 before supplementation to 4:1 after supplementation. These data illustrate that AA and DGLA reside in similar glycerolipid pools both before and after supplementation.

Influence of GLA supplementation on the release of fatty acids or the production of lipid mediators by stimulated neutrophils

Neutrophils were next obtained from subjects before and after supplementation and stimulated with ionophore A23187. The release of AA from the neutrophil glycerolipids after stimulation did not change following supplementation. However, the release of DGLA increased by 63%, 65%, and 69% in those volunteers receiving 1.5 g, 3.0 g and 6.0 g/day GLA, respectively (FIG. 7). These data support the hypothesis that the fatty acid composition of the neutrophil glycerolipids impacts on the fatty acids released upon cellular stimulation. They also suggest that the PLA_2 isotype(s) enzyme responsible for mobilizing fatty acids hydrolyzes DGLA in addition to AA.

While the aforementioned studies demonstrated that GLA supplementation did not influence the ex vivo release of AA from neutrophil glycerolipids, it was unclear whether GLA supplementation would alter leukotriene biosynthesis. To examine this question, neutrophils were stimulated and the synthesis of LTB_4, 20-OH LTB_4, and the 6 trans isomers of LTB_4 were measured by reverse phase HPLC analysis. Neutrophils from subjects supplementing their controlled diets with 3.0 g/day GLA produced 60% less LTB_4 than the same subjects before supplementation (FIG. 8). 20-OH LTB_4, 6-trans LTB_4 and 6-trans 12-epi LTB_4 levels were decreased to a similar degree after supplementation.

A final set of studies measured changes in the capacity of neutrophils to generate PAF ex vivo before and after GLA supplementation. Neutrophils of subjects

receiving 3.0 g/day of GLA produced 40% less PAF after supplementation than neutrophil obtained from those same subjects before supplementation. Taken together, these data reveal that GLA supplementation can alter the capacity of neutrophils to generate lipid mediators. This inhibition appears to occur at some step distal to the phospholipase-catalyzed cleavage of AA from membrane phospholipids.

Example 2

In Vitro Studies Examining The Metabolism Of GLA In Human Neutrophils

It is generally assumed that the liver has a key role in the in vivo elongation and desaturation of n-6 fatty acids. However, the role of other cells (especially inflammatory cells) and tissues has not been extensively studied. In addition, it is critical to evaluate the mechanism of leukotriene inhibition in less complex (than in vivo model) systems. To begin to address these problems, the inventor developed a model in which neutrophils could be incubated long-term with fatty acids or other fatty acid derivatives. Human neutrophils have been isolated and cultured overnight in RPMI, 2% insulin-transferrin and fetal bovine serum (FBS). In initial studies, varying concentrations of GLA (complexed to albumin) were provided to these cultured neutrophils for 24h. FIG. 10 shows quantities of DGLA and AA in neutrophils at increasing concentrations of GLA. The quantity of DGLA in neutrophil glycerolipids increased as a function of the concentration of GLA. In contrast, there was no change in the quantity of AA in neutrophil phospholipids. These data revealed that neutrophils have the capacity to take up GLA and rapidly elongate it to DGLA. However, they do not desaturate DGLA to form AA. These data are consistent with in vivo findings that indicate that GLA supplementation leads to an increase in DGLA, but not GLA or AA in human neutrophil glycerolipid. Furthermore, they provide direct evidence that the neutrophils themselves can elongate GLA in vivo.

It has been long recognized that arachidonate is hydrolyzed from membrane glycerolipids by phospholipase A_2 isotypes during cell stimulation. However, to date, there is little direct evidence that similar mechanisms exist to mobilize DGLA. To examine this question, neutrophils that had been cultured with varying concentrations of GLA (0 to 200 nmol) were stimulated with ionophore A23187, and mobilized fatty acids were measured by NICI GC/MS. DGLA along with AA were released from neutrophils during stimulation. To determine if neutrophils can further metabolize

DGLA to oxygenated products, stimulated cells were provided [^{14}C]-DGLA and products were measured by reverse HPLC. Neutrophils primed with LPS followed by stimulation with FMLP also converted DGLA to 15 HETrE. FIG. 11 illustrates that A23187 stimulated neutrophils produce a labeled product that migrated with 15-HETrE. In contrast, none of this product was observed in unstimulated cells. To the inventor's knowledge, these are the first studies to demonstrate the capacity of neutrophils to release DGLA and convert it into oxygenated products.

It is contemplated that neutrophils may also produce 8-hydroxy-9,11,14 eicosapentaenoic acid from DGLA. Borgeat and colleagues reported this to be a product of the incubation of dihomogammalinolenic acid with rabbit neutrophils. Studies were also designed to examine whether 15-HETrE produced by neutrophils might influence LTB_4 generation. Previous studies by Vanderhoek and colleagues have demonstrated that the AA product, 15-HETE, can reduce 5-lipoxygenase activity (Vanderhoek *et al.*, *J. Biol. Chem.*, 255:10064-10066, 1980). Neutrophils were isolated from normal unsupplemented volunteers and were treated with various concentrations of 15-HETrE and then stimulated with ionophore A23187. FIG. 12 shows the generation of LTB_4 and its major metabolite 20-OH LTB_4 by stimulated neutrophils. 15-HETrE induced a dose dependent inhibition of leukotriene generation with an IC_{50} of approximately 5 μM. In addition, DGLA at higher concentrations (IC_{50}, ~10 μM) also inhibited leukotriene generation. Although these studies do not prove that 15-HETrE or DGLA is the in vivo inhibitor of 5-lipoxygenase, they reveal that DGLA and oxygenated products of DGLA can potently influence eicosanoid generation.

Example 3

Influence Of The Combination Of GLA And Eicosapentaenoic Acid (EPA) On The Fatty Acid Composition Of Serum And Neutrophil Lipids

As mentioned above, a concern with the long-term effects of GLA supplementation is that there is an increase in serum levels of AA. There is a need, therefore, to find dietary strategies that will produce natural antagonist of AA in inflammatory cells without increasing serum AA. Previous in vitro studies in isolated hepatocytes and in vivo studies in animals suggest that EPA is a product inhibitor of the Δ^5 desaturase (Gronn *et al.*, *Biochim. Biophys. Acta*, 1125:3543, 1992; Dang *et al.*, *Lipids*, 24:882-889, 1989). In order to determine whether EPA would perform a

similar function in humans in vivo, three subjects on control diets (25% fat) were supplemented with a combination of EPA (1.5g/day) and GLA (3.0g/day) for three weeks. It was shown (FIG. 4 and FIG. 5) that this quantity of GLA (alone) induces marked increases in serum AA in both the short (3 weeks) and long term (12 weeks). The combination of GLA and EPA resulted in marked increases in GLA, DGLA and EPA in serum lipids. However, in contrast to the GLA supplementation alone, the combination of EPA with GLA did not cause an increase in serum AA (FIG. 13). These interesting results suggest that it may be possible to block the Δ^5 desaturase in humans with EPA thereby providing a means to supplement humans with high levels of GLA without concomitant increases in serum AA levels.

Example 4

In vitro Studies Examining the Metabolism of Stearidonic Acid in Human Neutrophils

As described above, human neutrophils (in vitro in overnight culture) will take up GLA and elongate it to DGLA but not further desaturate that DGLA to AA. An alternative route to depleting AA in neutrophils may also be useful in modulating the inflammatory responses mediated by AA and its metabolites. It was contemplated that the n-3 fatty acid, stearidonic acid (18:4) would also be elongated in neutrophils to form ω-3 arachidonic acid (FIG. 1). Varying concentrations of stearidonic acid were provided to cultured neutrophils for 24 h. Lipids were extracted and the quantities of fatty acids determined after base hydrolysis using GC/MS. There was no detectable (ω-3 arachidonic acid in neutrophils before supplementation (FIG. 14). However, addition of stearidonic acid caused a dose-dependent increase in ω-3 arachidonic acid in glycerolipids of these cells. In contrast to this increase, there was no increase in the Δ^5 desaturase product of ω-3 arachidonic acid, eicosapentaenoic acid, nor was there an increase in AA. Analogous to supplementation with GLA, these data reveal that neutrophils have the capacity to take up stearidonic acid and rapidly elongate it to ω-3 arachidonic acid. However, they do not further desaturate ω-3 arachidonic acid to form eicosapentaenoic acid.

These studies raise the interesting possibility that high levels of the AA analog, ω-3 AA, can be induced in inflammatory cells by providing inflammatory cells (in vitro or in vivo) with stearidonic acid. Moreover, they point out the potential

for (ω-3 AA to compete with natural AA (n-6) for enzymes (phospholipase A2 isotypes, cyclooxygenase isotypes, and 5-lipoxygenase) that convert AA to oxygenated metabolites.

Example 5

Development Of A Model To Study The Influence Of Diet On Clinical And Biochemical Parameters Of Asthma

Asthma presents a defined inflammatory disease that can be used as a model to test the efficacy of dietary manipulation. To this end an asthma model in humans was developed to test the reproducibility of the in vitro data and to determine the best dietary strategies. Another benefit of such a model is it allows the investigator to establish the effect of antigen challenge on AA levels in bronchoalveolar lavage fluid (BALF). Thus the present example teaches the use of an asthmatic model to test these parameters.

To this end, measures of airway physiology and analysis of BALF cellular and biochemical constituents were obtained from 5 stable atopic asthmatics before and after antigen challenge, both with and without prior corticosteroid therapy. A systemic corticosteroid arm was felt to be an impost to validate the physiologic variables as well as to ascertain which components in the BALF were sensitive markers of steroid-responsive inflammation. Additionally, AA levels were measured in BALF 4 h after inhaled antigen challenge (7 subjects) and at the time of the LAR (5 subjects). For comparison, identical BALF analyses were performed in ten normal volunteers (without antigen challenge or corticosteroids).

Study Design

Asthmatic subjects were defined using criteria proposed by the American Thoracic Society, *Am. Rev. Respir. Dis.*, 136:225-244, 1987. Normal subjects were healthy non-smokers, without respiratory symptoms. In all subjects, demographic data, history and physical examination, baseline spirometry, skin testing and methacholine PC20, using a tidal breathing technique, were obtained after informed consent for study participation. This was followed, no earlier than 7 days later, by baseline bronchoscopy for collection of BALF. This concluded the study protocol for normal subjects.

In 5 subjects, inhaled antigen challenge was performed using a previously described protocol and physiologic data collected. Not less than 2 weeks later, PC20 was again determined and antigen challenge repeated with BALF collected at the time of the LAR as determined during the first challenge. Two to 4 weeks later, these subjects were placed on 40 mg of prednisone daily for 7 days. Inhaled antigen challenge was again performed and BALF obtained at the same time after antigen challenge as on the previous visit. In an additional 7 subjects, BALF was obtained 4 h after inhaled antigen challenge, but without a subsequent course of prednisone therapy.

Statistical analysis

In the asthmatic patients, the changes in cell composition, eosinophil cationic protein (ECP), and protein in BALF among study conditions were examined using one way ANOVA with study period as the independent variable. If a significant interaction was found, a paired t-test was used to compare the means among test periods. Because the AA levels were not normally distributed within the groups, the non-parametric Wilcoxon signed-ranks test was used to analyze the differences in these measurements. A $p < 0.05$ was used to determine statistical significance.

Results

Measures of airway response to antigen challenge were consistent and reproducible both immediately and at LAR. The mean time for LAR, was 6.4 ± 1.5 h after challenge. The mean (± SD) fall in FEV_1 immediately after antigen challenge was 35 ± 8% while the fall at LAR was 28 ± 18% from baseline FEV_1. Following prednisone, both the immediate response and LAR were ablated.

The percentages of neutrophils and eosinophils in BALF were significantly higher in the asthmatics. The level of ECP rose after antigen challenge and was suppressed by corticosteroid administration ($p = 0.03$). While the percentage of eosinophils tended to mirror the changes in ECP, these changes did not achieve statistical significance. AA levels in BALF rose after antigen challenge (mean ± SE: baseline = 2.2 ± 0.3 ng/ml BALF; post-challenge = 3.9 ± 1.0; $p < 0.05$).

Discussion

This antigen challenge model of asthma provides reproducible physiologic (pulmonary function) data within and between subjects. Further, ECP appears to be a reproducible surrogate measure of eosinophil presence and/or activity in this model. In addition, AA levels can be observed to increase after antigen challenge in this model. Collectively, these measures offer the capability of assessing the efficacy of dietary manipulation with the expectation that significant differences among treatment regimens can be detected with a relatively small number of study subjects.

Example 6
Effect of GLA Supplementation on Eosinophil Fatty Acid Composition and Airway Functions

An issue with the GLA data obtained with neutrophils is its relationship to atopic asthma and, in particular, whether the neutrophil has a key role in atopic asthma. While there is evidence that the neutrophil has a role in atopic asthma, previous studies, to date, point to the eosinophil as having a central role. Therefore, it was important to determine how GLA was metabolized by human eosinophils. Thus, eosinophils were isolated from atopic subjects and incubated with GLA as described above. Like the human neutrophils, supplementation of human eosinophils resulted in a marked increase in DGLA but no change in the quantity of AA in eosinophil glycerolipids (FIG. 15). These data reveal that eosinophils have the capacity to take up GLA and rapidly elongate it to DGLA. However, eosinophils do not further desaturate DGLA to form AA.

In a second set of studies, two atopic asthmatics were recruited and challenged with antigens as described above. In both subjects, the concentration of antigen necessary to drop FEV_1 by greater than 20% was established. At a subsequent date, they were each challenged with these respective concentrations of antigen and monitored with spirometry to assess the development of an early and late responses. Each of these subjects were then placed on GLA supplementation for four weeks and then challenged again with the same dose of antigen. The subjects were then placed back on their normal diets for two weeks and then challenged again with the same respective dose of antigen. FIG. 16 shows the average of the responses of the two subjects at the three challenge periods. The magnitude of the early response was diminished (when compared to pre -and post GLA supplementation) in both subjects

four weeks after GLA supplementation. In contrast, GLA supplementation did not influence the late response.

Additionally, the influence of GLA supplementation in a human model of atopic asthma, on eicosanoid production, bronchial reactivity, and airway cellular influx can be measured as detailed herein. A random order, placebo-controlled crossover design preceded by a control diet "run in" phase study is performed. A crossover design is chosen to keep the number of subjects required for statistical validity as small as possible by minimizing the influence of intersubject variability with regards to the severity of asthma, environmental triggers and exposures, and nature and severity of the late asthmatic response (LAR). Subjects are studied after 3 weeks of a controlled "normal" diet with 25% of calories from fat, after 3 weeks of the "experimental" diet consisting of the "normal" diet supplemented with 4.5 grams (15 capsules/day) of GLA as borage oil, and after 3 weeks of a "placebo" diet consisting of the "normal" diet with 4.5 grams (15 capsules/day) of olive oil. Olive oil is 70% oleic acid, 13% C16, and 15% C18, (<1%, n-3) fatty acids as triglycerides. Neither oil supplement has either an odor or a taste when in capsule form. The experimental and placebo diets are given in random order. Each 3 week period is separated by a 4-6 week usual diet "washout" period when the diet of the study subjects is not controlled. Preliminary data from this group suggests that 4 weeks is a sufficient time period for abolition of an effect of diet during the preceding study period.

Results

It is contemplated that GLA supplementation and not placebo or "normal" diets will mitigate the response to antigen challenge as measured by the decrements in FEV_1 both immediate and the LAP, and reduce the influx of eosinophils into airways during the LAR. GLA supplementation will also likely attenuate antigen-induced urinary LTE_4 exertion and BALF AA increases.

While the antigen challenge model is capable of detecting a therapeutic effect due to prednisone with a small number of subjects, GLA supplementation may be associated with smaller, though significant, effects that are overlooked using relatively small sample sizes. The trial uses 10 subjects per group. Sample sizes are based on variance estimated and differences reported in the preliminary results. The

contemplated sample sizes have a 90% power to demonstrate an effect on pulmonary function (FEV_1) that is at least half the magnitude observed with oral prednisone therapy in the pilot study, at an alpha of 0.05. Asthma is a complex disease process and it is possible that significant effects in some components may be missed by using a model that is not sensitive to these effects. For example, an antigen challenge model would not be the appropriate system in which to detect an impact on neurally-mediated immediate processes (e.g., airway cooling). The effect of GLA supplementation, would, however, suggest that this antigen challenge model is appropriate.

Example 7

Dietary Strategies in Humans Utilizing Endogenous Elongase Activity Within Inflammatory Cells to Synthesize Structural Analogs of AA from Dietary Precursors Without Concomitantly Increasing Levels of Circulating AA

The data suggest there may be two strategies that can be utilized in humans to synthesize analogs of AA in inflammatory cells without concomitant increases in serum AA. The first approach (FIG. 17A) is to supplement the diets of humans with a combination of gammalinolenic acid (GLA) and a Δ^5 desaturase inhibitor such as eicosapentaenoic acid (EPA), for example. This strategy is based on in vitro data in hepatocytes and in vivo data in animals which indicate that EPA is a product inhibitor of the enzyme activity that synthesizes it, the Δ^5 desaturase (Gronn *et al.*, 1992; Dang *et al.*, 1989). The inventor has shown in two volunteers that administering of GLA in combination with EPA will induce a marked accumulation of DGLA in circulation and neutrophil lipids without causing a marked accumulation of AA in serum lipids (which is seen with GLA supplementation in the absence of EPA).

If in vivo administration of EPA is an effective means to block the hepatic Δ^5 desaturase, this combination should furnish a means to provide high concentrations of GLA to humans to synthesize the close structural analog of AA, DGLA, in inflammatory cells. This will have the action of inhibiting AA metabolism and eicosanoid biosynthesis, and attenuating signs and symptoms of inflammatory disorders, without the significant side effect of the accumulation of AA in circulation.

The second approach involves administering the n-3 fatty acid, stearidonic acid, to humans (FIG. 17B). This fatty acid is converted (by the endogenous elongase in inflammatory cells) to a structural analog of AA, (ω-3 AA and this product will

block AA metabolism and thus have anti-inflammatory effects. There have been several studies over the last few years that have examined the effects of in vivo supplementation with alpha linolenic acid (18:3, n-3) in both humans and animals. Generally, these studies have shown that alpha linolenic acid has only modest anti-inflammatory effects (Nordstrom *et al.*, *Rheumatol. Int.*, 14:231-234, 1995; Larsson-Backstrom *et al.*, *Shock*, 4:11-20, 1995; Clark *et al.*, *Kidney Int.*, 48:475-480, 1995; Shoda *et al.*, J. Gastroenterol., 30(suppl 8):98-101, 1995). However, only a very small portion of alpha linolenic acid is converted to stearidonic acid by the Δ^6 desaturase. In fact, this step appears to be the rate-limiting step in n-3 polyunsaturated fatty acid biosynthesis. As described herein, stearidonic acid supplementation is an efficacious means to block AA metabolism because it bypasses the rate-limiting step (Δ^6 desaturase) and is directly utilized by inflammatory cell elongase activity. A major advantage of stearidonic acid verse GLA (alone) as a supplement is that the elongation/ Δ^5 desaturase product from this precursor is EPA and not AA. Consequently even if EPA accumulates in serum components, it will not have the potential detrimental effects of AA.

Example 8

Inhibition of Delta-5 Desaturase by Eicosapentaenoic Acid in Human Liver Cells

The use of Δ^5 desaturase inhibitors in the practice of the present invention rests, in certain aspects, on the ability of those inhibitors to affect Δ^5 desaturase activity in the hepatic cells of a subject who is receiving GLA or DGLA as a dietary supplement, or especially as a treatment for an inflammatory disorder or condition, for example. As is described elsewhere herein, the DGLA, if taken up by liver cells, or GLA that has been elongated to DGLA undergoes Δ^5 desaturation in hepatic cells to produce arachidonic acid. This desaturation does not occur in immune system cells such as neutrophils, which lack the Δ^5 desaturase activity.

In order to demonstrate that an inhibitor such as eicosapentaenoic acid has the capacity to block Δ^5 desaturase, various concentrations of EPA were added exogenously, along with DGLA, to a human liver cell line, HEP-G2, and the conversion of DGLA to AA was monitored. The results are shown in Figs. 18A and 18B. The data indicate that EPA caused a dose-dependent inhibition of DGLA

conversion to AA with a maximum of 50% inhibition at 50μM. This study illustrates the effectiveness of adding a Δ^5 desaturase inhibitor in conjunction with GLA in order to reduce serum arachidonic acid.

Example 9

Determination of the Accumulation of ω-3 AA from Stearidonic Acid Treatment of Neutrophils Affects the Capacity of Cells to Release AA and Synthesize Eicosanoids

In additional work, the inventor has also demonstrated that human neutrophils rapidly take up stearidonic acid (18:4, n-3) and convert it to ω-3 AA. ω-3 AA is a 20 carbon fatty acid that is a close structural analog of AA (n-6). Thus, ω-3 AA may also serve as a competitive antagonist for AA (n-6) during AA metabolism. The following examples provide details of procedures used to investigate this strategy.

It is not known whether ω-3 AA-containing phospholipids will influence the capacity of PLA_2 isotypes to release AA (n-6) in stimulated neutrophils or whether ω-3 AA will effect enzymes distal to phospholipase A_2 such as 5-lipoxygenase or cyclooxygenase I and II. These issues are readily explored by 'loading' human neutrophils in vitro with ω-3 AA; and then activating the cells. The capacity of the cells to release AA, stearidonic acid, and ω-3 AA, as well as produce eicosanoids, is examined.

Isolated neutrophils (20 million/40 ml of media) or eosinophils (10 million/40 ml of media) are maintained in culture with RPMI, 2% insulin transferrin, 1% FBS and various concentrations of stearidonic acid (quantities ranging from 0 to 200 nmol). After 24 h, these cells are washed (2x) with Hanks Balanced Salt Solution containing 0.25 mg/ml albumin and then resuspended at a concentration of 10 million/ml. Cells then are stimulated with ionophore A23187 (1 μM) and maintained at 37°C for an additional 5 min. For a more physiologic stimulus, neutrophils are incubated in 10% autologous plasma containing 1 μg/ml LPS for 30 min. Eosinophils are stimulated with PAF (1 μM). Cells are then washed and incubated with or without FMLP (1 μM). Reactions are terminated with methanol/chloroform (2:1, v/v) or methanol, for fatty acid release or leukotriene analysis, respectively.

To determine the quantity of fatty acids released from glycerolipids during cell activation, octadeuterated AA and trideuterated stearic acid are added as internal

standards to the terminated reaction mixture and lipids are extracted by the method of Bligh and Dyer, 1959. Fatty acids in samples are then analyzed by NICI GC/MS. Quantities of leukotrienes are determined following reverse phase HPLC separation as described above. Quantities of prostaglandins are determined by NICI GC/MS. From these studies, it can be determined: 1) Whether the presence of ω-3 AA or stearidonic acid-containing phospholipid in cellular membranes of neutrophils and eosinophils influence the capacity of neutrophil phospholipase A_2(s) to mobilize AA (n-6); 2) Whether ω-3 AA or stearidonic acid is released from membrane glycerolipids during cell activation; and 3) Whether the presence of ω-3 fatty acids affects the capacity of neutrophils and eosinophils to synthesize leukotrienes and prostaglandins.

In addition to examining the effect of ω-3 arachidonic acid on AA metabolism, it is also determined whether ω-3 arachidonic acid itself is metabolized by neutrophils to eicosanoid-like products. In these studies, A23187-stimulated and unstimulated neutrophils are incubated with ω-3 arachidonic acid (from 1 to 50 μM for 10 min.). Products are then separated by reverse phase HPLC and fractions monitored at 234 nM [HETE-like compounds] or 270 [leukotriene-like compounds]. New products observed with ω-3 AA and A23187 addition (not observed with either alone) are isolated and converted to methoxime-pentafluorobenzyl-ester-trimethylsilyl ether derivatives as described previously. Derivatized products as carboxylate anions are analyzed by negative ion chemical ionization GC/MS. It is possible that some products of ω-3 AA may not absorb at the above mentioned wavelengths. In this case, there are several HPLC-electrospray mass spectrometry/mass spectrometry procedures for characterizing the double bond positions and position of hydroxyl modifications of fatty acids. These are used to definitively identify products from ω-3 AA.

It is likely that ω-3 AA attenuates the capacity of cells to synthesize leukotrienes. Further, neutrophil PLA_2(s) hydrolyzes ω-3 AA from cellular glycerolipids during cell activation.

Example 10

Effects of in vivo Supplementation with Oils Enriched in Stearidonic Acid (18:4, n-3) on the Quantities and Ratios of n-6 and n-3 Fatty Acid in Serum and Neutrophil Lipids and the Ex Vivo Capacity of Stimulated Neutrophils from Supplemented Volunteers to Release Fatty Acids and Produce Eicosanoids

It has been demonstrated that in vitro incubation (for 24h) of stearidonic acid with human neutrophils leads to a dramatic increase in the quantity of ω-3 AA in cellular glycerolipids and thus a large increase in the ω-3 AA/AA ratio in these complex lipids. These data indicate that the neutrophil elongase activity can be utilized to synthesize close structural analogs of AA from appropriate dietary precursors. These analogs are then postulated to affect AA metabolism via phospholipase A_2, 5-lipoxygenase or cyclooxygenase I or II.

It is contemplated that there is an in vivo dose-dependent relationship between the quantity of stearidonic acid consumed in diets and the quantities of stearidonic acid, ω-3 AA and eicosapentaenoic acid in serum lipids and ω-3 AA in neutrophil lipids. If (ω-3 AA accumulates in neutrophil lipids as predicted and it acts as a competitor with AA (n-6), then it is also likely that increasing stearidonic acid doses will correlate with a further attenuation of leukotriene generation by neutrophils and whole blood, and a concomitant increase in ω-3 AA release from cellular phospholipids.

Recruitment of subjects, diet preparations and monitoring diet compliance are all performed as described above. To limit variability of volunteers' normal diets, four randomly assigned groups of volunteers (10 per group, 5 males and 5 females) are provided identical 25% fat diets for two weeks before starting stearidonic acid (SDA) supplementation. Then one group of volunteers consumes 1.5 g SDA/day; another group consumes 3.0 g SDA/day and a third group consumes 6.0 g SDA/day. A separate (fourth) group of subjects consumes 3.0 g of alpha linoleic acid from Crossential GLA. Crossential GLA is a commercially available oil from Croda which contains >75% of its fatty acids as alpha linolenic acid. This oil contains no stearidonic acid. This control is necessary to test the hypothesis that bypassing the Δ^6 desaturase is necessary to effectively produce analogs of AA (ω-3 AA) in inflammatory cells. All groups consume their respective supplement and identical controlled 25% diets for four weeks. Fasting blood is collected before starting the 25% diet (before diet control) and one and seven days before starting the

supplementation. Subsequently, fasting blood samples are collected every 7 days after supplementation and 2 weeks after supplementation has ceased.

Analysis of fatty acids and eicosanoids in neutrophils and whole blood

Fasting (12h) blood samples are obtained at each of the time points (in all protocols) described above. The following fatty acid and eicosanoid measurements are made at each time point. Eicosanoid measurements in whole blood and in stimulated neutrophils are performed as described above.

Measurements of free AA, ω-3 AA and stearidonic acid in stimulated neutrophils

Stimulated neutrophils release AA from phospholipids utilizing PLA_2(s) reactions. It is also possible that PLA_2(s) recognizes ω-3 AA or SDA-containing phospholipids, or supplementation with SDA blocks the PLA_2-induced release of AA in neutrophils. Therefore, free AA, (ω-3 AA, SDA, and eicosapentaenoic acid are measured by NICI GC/MS before and after stimulation of neutrophils isolated from each volunteer at each dietary time point. Neutrophils are stimulated with ionophore A23187, LPS or LPS and FMLP.

It is expected that stearidonic acid (like GLA) is both elongated and Δ^5 desaturated in serum compartments to form ω-3 AA and eicosapentaenoic acid (EPA), respectively. It is also contemplated that only ω-3 AA accumulates in neutrophil glycerolipids, thus increasing the ω-3 AA/AA ratio. Stearidonic acid containing oils are also expected to induce much higher quantities of ω-3 AA in neutrophil lipids than alpha linolenic acid. It is likely that the accumulation of ω-3 AA translates into a reduction in the capacity of blood cells, the neutrophil in particular, to produce eicosanoids. Again, one of the major advantages of stearidonic acid versus GLA as a supplement is that the elongation/ Δ^5 desaturase product from this precursor is EPA and not AA. Consequently, even if EPA accumulates in serum components, it will not enhance AA metabolism.

Example 11.

A Preferred Embodiment of a Dietary Fatty Acid Supplement

A preferred composition is a stabilized emulsion that can be consumed neat or easily mixed in a drink or yogurt. The preferred composition of the emulsion is as follows:

Constituents	Weight (g)
Borage Oil (concentrated: 40 wt % GLA)	21.00
Marine Oil (concentrated: 33 wt % EPA)	16.5
Lecithin	0.50
Flavor and Flavor Masking Agent	2.00
Colorant	0.05
Ascorbyl palmitate	0.20
Sorbic acid	0.16
Sucrose	25.00
Xanthan gum	0.3
Water	29.29
Glycerin	5.00
Total	100

The composition is preferably packaged in an oxygen-free environment in single daily dosage packages made of oxygen impermeable materials such as foil-lined pouches. The recommended daily dosage of 20 grams per day would deliver about 1.5 grams of gammalinolenic acid and about 1.0 gram of eicosapentaenoic acid per day. The formulations preferably contain natural anti-oxidants, natural fruit flavors and natural coloring agents. The stabilized emulsion also may contain a natural sweetener and natural preservative.

Example 12.

Administration of the Dietary Supplement of the Invention Inhibits Leukotriene Biosynthesis

A streamlined pharmaceutical approach was used to examine the pharmacokinetics and metabolism of the active ingredients, GLA and EPA, of complex oils, the metabolic interactions between these active ingredients and the minimal dosage and optimal ratios of these components required to block whole blood ex-vivo leukotriene biosynthesis. To optimize the bioavailability of these fatty acids, an emulsion formulation was developed resulting in a safe and efficacious novel product combination that enhances pharmaceutical regimens currently used in treating inflammatory disorders such as asthma, allergic rhinitis and atopic dermatitis.

Design of Clinical Trials:

Trials 1 and 2 were performed at the General Clinical Research Center (GCRC) at Wake Forest University Medical Center (WFU). All protocols were approved by the Institutional Review Board at Wake Forest University Medical Center, and each subject gave informed consent before beginning the study. Trial 3 was conducted at the Quintiles Phase I clinical trials facility in Lenexa, Kansas. The protocol was approved by an external Institutional Review Board, and each subject gave informed consent before beginning the study.

Trial 1: Determination of the minimal dose of GLA required to reduce leukotriene synthesis

Previous studies investigating the effect of dietary GLA on the capacity of isolated human neutrophils to synthesize leukotrienes have involved the consumption of large quantities of GLA (3-6g per day) (*Barham et al., J. Nutr.* 2000;130(8):1925-31; *Johnson et al., J. Nutr.* 1997;127(8):1435-44). Trial 1 was performed to determine the minimum effective dose of GLA required to decrease *ex-vivo* leukotriene biosynthesis in stimulated whole blood.

Human volunteers (n=10) reported to the nursing station at the WFU-GCRC at scheduled times and were assigned to one of two groups. Vital signs were obtained and baseline measurements of plasma fatty acid concentrations and stimulated whole blood leukotrienes were obtained. Additionally, platelet aggregation, total serum

cholesterol, HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C) and circulating triglyceride concentrations were measured. Subjects assigned to Group 1 (n=5) were instructed to consume concentrated borage oil capsules delivering 1.5g GLA/day, and subjects assigned to Group 2 were instructed to consume concentrated borage oil capsules delivering 0.75g GLA/day, for 3 weeks. The fatty acid composition of the supplements is given in Table 1 of the present example. Subjects were instructed to maintain their normal dietary habits during the study and to return to the GCRC weekly for vital sign assessment and to monitor the same laboratory values that were assessed at baseline. Subjects were also questioned by the nursing staff about their general well-being. Washout values of all measurements were then obtained two weeks after cessation of supplementation.

Table 1: Fatty acid composition of daily supplements consumed by subjects in all clinical trials.

	Fatty Acid Composition (% of Total)				
	Trial 1	Trial 2		Trial 3	
Fatty acid	Borage Oil	Borage Oil + Low n-3	Borage Oil + High n-3	PLT3514 Emulsion	Olive oil Emulsion
16:0	7.96	7.10	5.87	5.96	11.40
16:1	0.07	0.27	0.63	0.51	1.00
18:0	3.94	3.89	3.84	2.91	3.05
18:1	12.21	12.55	12.13	9.59	76.50
18:2	26.71	22.12	15.87	15.0	6.71
18:3 n-6	47.65	36.84	25.61	28.6	0.15
18:3 n-3	0.13	3.19	2.46	0.30	1.10
18:4 n-3	N/D	1.32	0.92	N/D	N/D
20:4 n-6	N/D	0.06	0.17	0.98	N/D
20:3 n-3	N/D	N/D	0.06	0.07	N/D
20:4 n-3	N/D	0.28	0.75	1.01	N/D
20:5	0.35	6.39	16.64	15.40	N/D
22:1	0.25	0.24	0.26	2.32	0.09
22:4	N/D	0.08	0.15	0.11	N/D
22:5 n-3	0.05	0.95	2.47	1.89	N/D
22:6	0.25	4.23	11.00	8.97	N/D

Trial 2: Determination of the minimal dose of EPA necessary to reduce plasma AA levels

Previous human trials in which subjects' diets were supplemented with GLA-containing oils reported an increase in plasma AA levels (*Barham et al., J. Nutr.* 2000;130(8):1925-31; *Johnson et al., J. Nutr.* 1997;127(8):1435-44). Barham and

colleagues reported that this dietary GLA-induced increase in plasma AA concentrations could be prevented when subjects consume large quantities of n-3 PUFA containing oils (3g of EPA per day). The present trial was performed to determine the minimum effective dose of dietary n-3 PUFA required to prevent the increases in plasma AA levels associated with the daily consumption of oils containing GLA.

Twenty human volunteers reported to the nursing station at the WFU-GCRC at scheduled times and were assigned to one of two groups of ten subjects each. After obtaining vital signs and clinical laboratory measurements, all subjects were instructed to supplement their normal diet for 3 weeks with concentrated Borage oil capsules delivering a dose of 1.5g GLA per day. Subjects assigned to Group 1 also supplemented their diet with 1.25g per day of concentrated n-3 PUFA-enriched oil capsules (0.7g/day n-3 PUFA containing 0.25 g EPA). Subjects assigned to Group 2 also supplemented their diet with 3.5g per day of concentrated n-3 PUFA-enriched oil capsules (2.2g/day n-3 PUFA containing1.0 g EPA). The total fatty acid composition of the daily supplementation is given in Table 1. All clinical and laboratory measurements followed the exact same schedule as that described in Trial 1 above.

Development of an oral emulsion enriched in PUFA

The results of trials 1 and 2 revealed the dosages of GLA and n-3 PUFA delivered in gelatin capsules required to modulate LT biosynthesis and plasma fatty acid composition. In subsequent trials, an oil-in-water emulsion was prepared containing approximately 21 % concentrated Borage oil and 16.5 % concentrated marine oil. This allowed individuals to consume these dietary lipids in a once a day dosage form. The oral bioavailability of fatty acids in this formulation was found to be superior to that of gelatin capsules as described hereinbelow. The fatty acid composition of this emulsion is provided in Table 1 above. According to a preferred embodiment a 10g dose of this emulsion would provide .75g of GLA and .43 g of EPA.

Trial 3: Safety, efficacy and pharmacokinetics of fatty acid emulsion

A single center, inpatient, randomized, double-blind, parallel, placebo-controlled, escalating-dose trial was performed to evaluate the safety and tolerability

of three doses of the above-described emulsion in healthy adult subjects. In addition, the kinetics of the appearance of EPA and GLA in plasma and the effect of PLT 3514 on whole blood LTB_4 biosynthesis were measured. Subjects who qualified for the study were enrolled and were administered either 10g, 20g or 100g of the emulsion of the invention or of an emulsion containing 37.5% olive oil (placebo) daily for 14 days. The emulsions were dissolved in 250 mL orange juice and consumed immediately prior to breakfast.

For pharmacokinetic assessments, on day 1 and day 14 serial blood samples were collected at predose and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, and at 24 hours following the dose. In addition, trough pharmacokinetic blood samples were obtained predose on day 7 through day 13. Stimulated whole blood LTB_4 biosynthesis was measured at predose on days 1 and 14. Cell Blood Counts (CBC) were also measured predose, on days 1 and 14 while platelet aggregation tests using collagen as an agonist were conducted at predose on days 1, 7, and 14.

Safety assessments were performed at screening and throughout the study. A panel of clinical laboratory measurements, vital signs and 12-lead electrocardiogram were evaluated at screening and at post study. In addition, vital signs and abbreviated hematology laboratory tests (hematocrit and hemoglobin) were obtained on days 0 and 7, and serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvate transaminase (SGPT) were measured on days 1, 5, 7 and 10 in addition to the screening and post-study evaluation.

II. Materials and Methods:

The following materials and methods are provided to facilitate the practice of Example 12:

Materials. Concentrated Borage oil (40% GLA), concentrated fish oil (67% n-3 fatty acids, 33% EPA), Echium oil (45% n-3 fatty acids) and olive oil capsules were supplied by Croda Leek Ltd. (Goole, East Yorkshire, Great Britain). Zymosan A from Saccharomyces cerevisiae was purchased from Aldrich (Milwaukee, WI). Human serum for the preparation of opsonized zymosan was prepared from fresh blood drawn from healthy volunteers. Sep-Pak C18 cartridges (500mg, 2 mL) were purchased from JT Baker (Phillisburgh, NJ) or Waters (Milford, MA). Sodium

heparin 143 USP Vacutainer 16x100mm tubes (JT Baker) were used for blood collection. Hank's Balanced Salt Solution (HBSS) was purchased from HyClone (Logan, UT). Prostaglandin B_1 (PGB_1), 19-hydroxy(OH)-PGB_2, 20-carboxy(COOH)-LTB_4, 20-OH-LTB_4 and LTB_4 were from Cayman Chemical Co. (Ann Arbor, MI). A modified Luna C-18 HPLC column, 5μm pore size and 250mm length x 2 mm ID was purchased from Phenomenex (Torrance, CA). 1,2-Diheptadecanoyl-sn-glycero-3-phosphorylcholine (DHDPC) was purchased from Matreya (Pleasant Gap, PA) and octadeuterated arachidonic acid ($^2H_8$-AA) was obtained from Biomol Research Laboratories Inc. (Plymouth Meeting, MA).

Fatty acid profiling. Levels of plasma fatty acids were determined as indicated either by negative ion chemical ionization gas chromatography mass spectrometry (NICI-GC/MS) as previously described (*Surette et al., Biochem.* 1996;35(28):9187-96) or by GC with flame ionization detection (GC-FID). For plasma fatty acid profiling by GC-FID, lipids were extracted from 100 μl platelet-free plasma by the method of Bligh and Dyer (*Bligh, Canadian J. Biochem. Physiol.* 1959;37:911-917). The internal standard DHDPC (40μg) was added to the monophase before organic solvent extractions. Base hydrolysis and derivatization was then carried out on the extracted lipids using methanolic sodium hydroxide and boron trifluoride. Methyl esters were extracted with hexane and 23% NaCl solution. Samples were then injected onto Finnigan-Thermoquest GC (Austin, TX) equipped with an AS2000 autosampler and an FID detector. The column was a DB-23 (30 m length, 0.25 mm film thickness and 0.25 mm ID) from J&W Scientific (Folsom, CA). Helium was the carrier gas and nitrogen was the make-up gas. Hydrogen and air were the fuel gas for the FID. The oven temperature was increased at a rate of 3^0C/min from an initial temperature of 160^0C to 230^0C. Injector and detector temperatures were both operated at 250^0C. The injection split ratio was 50:1 and 1 μl of sample was injected.

Serum cholesterol and triacylglycerides. Total serum cholesterol was determined colorimetrically using the Roche Molecular Biochemicals (Indianapolis, IN) Diagnostics Cholesterol HP reagent according to the manufacturer's instructions. Serum HDL-C levels were determined using the colorimetric EZ HDL™ assay (Sigma Chemical Co., St. Louis, MO) according to the manufacturer's instructions. Serum

triacylglycerides (TG) were determined using the Technicon RA systems triglyceride method (Miles Inc., Tarrytown, NY). Serum LDL-C content was calculated based on the method of Friedewald and colleagues (*Friedewald et al., Clin. Chem.* 1972;18(6):499-502).

Platelet aggregation. Platelet aggregation was monitored in response to the agonists adenosine diphosphate, thrombin and collagen using an aggregometer.

Stimulation of blood for leukotriene biosynthesis. Blood was drawn in vacutainer tubes containing heparin. Duplicate 1 mL aliquots of blood were stimulated for 30 minutes at 37^0C with opsonized zymosan (5mg/mL final concentration) as previously described [*Surette et al., Anal. Biochem.* 1994;216(2):392-400). To stop the reaction, samples were centrifuged at 5^0C for 10 minutes at 914xg and plasma (200 µl in duplicate) was carefully removed and transferred into 1.2 mL of methanol containing 50 ng PGB_1 and 50 ng 19-OH-PGB_2 (internal standards). Samples were then stored at -20^0C overnight. The next day samples were centrifuged at 914 x g for 10 minutes, supernatant fluid was decanted into plastic tubes and acidified water (0.1% v/v acetic acid) was added resulting in a 10% final methanol content. Samples were then loaded on C-18 Sep-Pak columns pre-conditioned with 2 mL methanol and regenerated with 3 mL acidified water. Samples were washed with 2mL acidified water followed by 2 mL hexane and were eluted with 4 mL methanol. Solvents were evaporated under a stream of nitrogen and samples were then resuspended in 150 µl methanol, vortexed for 10 seconds and diluted with 350 µl Milli-Q water prior to injection on the HPLC column.

Leukotriene Analysis. An HPLC gradient method was used with a C-18 Luna column and a starting (A) solvent comprised of methanol:water (50:50, v/v) and a final solvent (B) comprised of acetonitrile:methanol (70:30, v/v). Both mobile phases were acidified with 0.2% acetic acid. A step-wise gradient was used: B was increased for the first 15 minutes at 4%/min and followed by a 13%/min gradient. The final solvent was held at 100% for 5 minutes and the column was then regenerated with A for 17 minutes. Levels of LTB_4 and its ω-oxidation products, 20-COOH-LTB_4 and 20-OH-LTB_4, were determined using a diode array detector monitoring UV absorbance from

260-320nm and with quantification at 280nm. When referring to LTB_4 in blood samples, it represents the sum of LTB_4 and its ω-oxidation products.

Statistical Analysis. Statistical analysis was performed using One-Way Anova analysis with t-test and Dunnet's test (JMP software, SAS institute, Cary, N.C.)

RESULTS

Dose and time-dependent effect of GLA on leukotriene B_4 production

Previous human trials have shown that the consumption of large amounts of dietary GLA reduces the capacity of isolated neutrophils to synthesize leukotrienes when stimulated with the calcium ionophore, A23187. The initial trial in this study was designed to determine the minimum daily dose of GLA required to affect LTB_4 production in whole blood stimulated with opsonized zymosan. This method of assessing the synthetic capacity for LTB_4 is thought to be more physiologically relevant than that used in previous trials where neutrophils were isolated, resuspended in a saline buffer and stimulated with the non-specific potent calcium ionophore A23187. In a whole blood assay the introduction of artifacts associated with cell isolation are minimized (*Surette et al., Mol. Pharmacol.* 1999;56(5):1055-62; *Krump et al., J. Exp. Med.* 1997;186(8):1401-6) and cells are bathed in their natural medium where cell-cell interactions are preserved thus allowing for the transcellular metabolism of arachidonic acid (*Fiore et al., J. Exp. Med.* 1990;172(5):1451-7; *Gronert et al., Methods Mol. Biol.* 1999;120:119-44). Subjects were administered 1.5g or 0.75g GLA daily for three weeks and stimulated blood leukotriene levels were monitored each week and two weeks post supplementation. FIG. 19A shows concentrations of LTB_4 from the blood of subjects consuming 0.75g or 1.5g of GLA over a three week period. When compared to pre-supplementation baseline values there was a significant decrease in LTB_4 biosynthesis in subjects consuming 1.5 g GLA daily. In contrast no significant change in LTB_4 was observed in subjects consuming 0.75g of GLA daily. FIG. 19B illustrates that a minimum of two weeks is required to achieve a significant decrease in leukotriene biosynthesis in subjects consuming 1.5 g GLA and this decrease was maintained throughout the three weeks of supplementation. The capacity to synthesize leukotrienes returned to baseline levels within two weeks of terminating supplementation.

Levels of DGLA and AA in plasma were also analyzed at weekly intervals. There was a significant increase in plasma DGLA (115±12 μmol/L at baseline; 206±17* μmol/L at week 1, *p<0.05 one-way Anova t-test) and in AA levels (378±48 μmol/L at baseline; 516±49* μmol/L at week 1, *p<0.05 one-way Anova t-test) after one week of dietary supplementation with 1.5g of GLA. The concentrations of these fatty acids did not further increase over the course of the supplementation period, but returned to pre-supplementation baseline levels following the 2-week washout period. On the other hand, no significant increases in plasma DGLA or AA concentrations compared to baseline were observed in subjects supplementing their diets with 0.75g/day of GLA for three weeks (data not shown). None of the subjects reported any adverse events in their general health or welfare during the course of these studies. No significant changes in serum total cholesterol, HDL-C, LDL-C or triglycerides were observed at any supplementation concentration or any time point throughout the study (data not shown). Since increased circulating AA levels have been shown to increase platelet reactivity, platelet aggregation was also monitored (*Seyberth et al., Clin. Pharmacol. Ther.* 1975;18(5 Pt 1):521-9). No changes in platelet aggregation in response to collagen, ADP and thrombin were observed during the course of the study (data not shown).

Dose-dependent effect of n-3 fatty acids on the in vivo conversion of ingested GLA to AA

In light of the potential adverse effects of high levels of plasma AA, it was important to block the in vivo conversion of ingested GLA to AA. Previous studies have revealed that the consumption of large quantities of n-3 PUFA prevent the increase of AA in plasma associated with the consumption of high levels of dietary GLA (*Barham et al., J. Nutr.* 2000;130(8):1925-31). The results in Trial 1 described above indicated that supplementation with 1.5g GLA per day was sufficient to inhibit stimulated blood LTB_4 biosynthesis. However, 1.5g/day GLA also resulted in a significant accumulation of AA in plasma (FIG. 20). Therefore, a second trial was designed to determine the minimum dose of n-3 PUFA required to eliminate plasma AA accumulation associated with consumption of 1.5g/day GLA. FIG. 20 illustrates that supplementation of n-3 PUFA as little as 0.7g/day blocked the accumulation of plasma AA associated with consumption of 1.5 g GLA/day. This prevention of AA

accumulation was accompanied by a significant increase in plasma DGLA and EPA levels compared to baseline (data not shown). These results define a range of dietary n-3 PUFA needed to prevent increases in plasma AA levels associated with the consumption of 1.5g per day of dietary GLA.

Development of an oral emulsion that enhances the bioavailability of ingested PUFA

A practical impediment to the ingestion of the high doses of PUFA required to affect leukotriene biosynthesis was the large number of gelatin capsules that needed to be ingested to achieve the efficacious dose. Therefore, an oral formulation was developed in accordance with this invention, with the goal of enhancing the bioavailability of PUFA thereby reducing the required daily dose. The oil-in-water emulsion was prepared containing 21% Borage oil and 16.5% marine oil to deliver 1.5g GLA and 1.0g EPA in a once a day 20g dosage form. Fig. 21 shows that there was a marked enrichment (approximately two-fold) of plasma EPA, GLA and DGLA in subjects consuming the emulsion when compared to subjects consuming identical doses of PUFA as gelatin capsules. These data revealed that the form in which the active fatty acids are delivered, has a significant impact on their bioavailability.

A rising dose safety and efficacy trial with an oral PUFA emulsion

Having defined a minimum effective dose of dietary GLA required to decrease LTB_4 biosynthesis in stimulated blood, as well as the range of dietary n-3 PUFA required to prevent the rise in plasma AA levels associated with the consumption of GLA, a third trial was designed to evaluate the safety, efficacy and pharmacokinetics of the oral PUFA emulsion described in the preceding section.

The trial was conducted in a Phase I clinical facility where a total of 47 housed subjects consumed either 10g/day, 20g/day or 100g/day of the aforementioned emulsion or a placebo emulsion (olive oil) for 14 days. Of the 42 subjects completing the study, 9 received daily doses of 10g, 17 received doses of 20g, 7 received doses of 100g and 9 received placebo. The five subjects who discontinued the study prematurely received study treatment as follows: One of the subjects received 10g per day for 4 days, one subject received 10g per day for 9 days and one subject received 20g of the PUFA emulsion of the invention for 12 days. These subjects withdrew due to family emergencies. Two subjects who had received 100g of the oral PUFA

emulsion of this invention for 7 and 10 days were withdrawn from the study due to an elevation in SGPT.

No meaningful changes in vital signs were observed from screening to post study. No differences in the occurrence of treatment emergent adverse events were measured between the groups receiving the PUFA emulsion and placebo except for the elevated SGPT discussed below. No changes in mean clinical laboratory values or in abbreviated hematology laboratory tests were observed throughout the study except for a 43% decrease in plasma TG measured in the 100g group (baseline 1.57 mmol/L, Day 14 value 0.89 mmol/L, $p<0.05$) and for mean SGOT and SGPT values which increased from baseline to day 10 (19.3 to 26.0 U/L) and baseline to day 14 (23.7 to 46.3 U/L), respectively, in the 100g group. A SGPT value of 102U/L was measured in one subject on day 7 and a value of 98U/L was measured in a second subject on day 10. These two subjects discontinued supplementation upon reaching SGPT values of 2x normal and were followed until days 22 and 39, respectively, at which time their SGPT values had returned to normal. A SGPT value of 64U/L was measured in a third on day 14 and rose to a value of 134U/L on day 16 but returned to the normal range by day 19. No other relevant clinical laboratory abnormalities or significant adverse events associated with supplementation were measured in these subjects.

Pharmacokinetic analysis was performed based on the repeated measurement of plasma EPA and GLA concentrations over the 24-hour period after the administration of the first dose of emulsion on day 1 and again after the administration of the last dose on day 14. FIG. 22A and FIG. 22B show the mean plasma concentrations of GLA and EPA during the 24-hour period after dosing with 20g of the PUFA emulsion of this invention on day 1 and 14. Plasma GLA and EPA levels rapidly increased with maximal concentrations observed within 3 hr of ingestion of the emulsion. After the initial 24-hour period, the fasting levels of both GLA and EPA were elevated in all treatment groups compared to the baseline concentrations indicating that tissue enrichment was achieved following one single dose. Table 2 shows the pharmacokinetic analysis of GLA and EPA on day 1 and 14 for these dosage groups.

Table 2: Pharmacokinetic parameters for the three daily dosing groups 10g, 20g and 30g in human subjects consuming oral PUFA emulsion

Day		10g n=11	20g n=18	100g n=9	10g	20g	100g
1	C_{max}(μg/mL)[a]	34.1±16.5	72.9±33.6	413.6±130.9	25.5±11.6	49.7±18.4	344.7±109.4
	T_{max} (hr)[a]	3.5±1.4	3.4±1.6	4.9±1.4	5.9±2.6*	4.7±1.8*	6.00±0.01*
	$AUC_{(0-z)}$ (hr.μg/mL)[a]	381±162	674±292	3127±787	392±119	645±241	3485±797
14	C_{max}(μg/mL)[a]	69.3±17.9	98.2±27.9	355.9±50.7	70.6±11.5	129.0±24.3	407.3±79.4
	T_{max}(hr)[a]	3.1±0.8	3.06±1.2	4.43±1.5	3.50±1.5*	3.91±1.7*	5.57±1.1*
	$AUC_{(0-z)}$ (hr.μg/mL)[a]	372±121	521±168	1859±383**	329±140	447±319	1542±1098**

a: Mean Values (± SD) presented

*significantly different from respective GLA group ($p<0.05$ paired t-test)

**significantly different from day 1 ($p<0.05$ paired t-test)

There was a dose-dependent increase in the C_{max} and Area Under the Curve (AUC) values for both GLA and EPA. The t_{max} values for GLA were significantly smaller than for EPA in all dosage groups. Differences in the position of these fatty acids on the triglyceride backbone could account for such differences (*Entressangles et al., Biochim. Biophys. Acta.* 1966;125(3):597-600; *Decker, Nutr.Rev.* 1996;54 (4 Pt 1):108-10). Interestingly, a significantly smaller AUC was measured on day 14 as compared to day 1 in the 100g dosage group. This observation is consistent with previous reports which demonstrated that the AUC of postprandial triglycerides is significantly decreased in subjects consuming diets supplemented with high levels n-3 PUFA (*Harris et al., Am. J. Clin. Nutr.*1993;58(1):68-74).

Representative daily fasting plasma concentrations of GLA and EPA for the 20g dose are presented in FIG. 22C. Trough levels of both fatty acids were achieved by day 7 in all dosage groups without further elevation in their concentrations for the remainder of the study. This indicated that steady state levels of GLA and EPA were achieved with no apparent cumulative effects for up to 14 days at all doses. The total plasma fatty acid concentrations for each group were also examined on days 1 and 14 (Table 3). In addition to EPA and GLA, there was a dose-dependent enrichment in the

concentration of several fatty acids including DGLA and docosahexaenoic acid (DHA).

Table 3. Fatty acid composition of plasma in normal subjects before and after two weeks of daily supplementation with 10g, 20g or 100g of oral emulsion per day.

Fatty acid	10g Group		20g Group (μmol/L)		100g Group	
	Day 1	Day 14	Day 1	Day 14	Day 1	Day 14
14:0	110±10	137±13*	168±15	156±18	81±26	83±11
16:0	2215±163	2337±103	2326±156	2314±188	2026±192	1377±78
16:1	160±14	161±16	211±28	161±28	170±38	68±13
18:0	956±35	1089±57	1022±40	1121±47	896±24	908±92
18:1 n-9	1830±156	1554±98	1791±132	1433±121*	1870±249	826±90*
18:1 n-7	165±13	138±10	145±10	112±9*	179±22	81±11*
18:2	2913±165	2463±165	2606±107	2090±134*	2653±331	1462±116*
18:3 n-6	45±4	94±5*	47±7	111±10*	32±3	161±11*
18:3 n-3	55±6	62±6	52±5	53±6	59±11	37±7
20:3 n-6	120±10	168±11*	114±7	184±13*	124±11	132±9
20:4 n-6	617±57	594±47	601±40	576±47	589±66	406±34*
20:5 n-3	29±4	114±3*	33±4	185±16*	22±2	374±27*
22:1	2±1	5±1*	5±2	8±2*	16±2	12±1*
22:4 n-6	20±2	20±1	20±2	17±2	20±1	6±0.3*
22:5 n-3	36±3	58±1*	29±3	78±8*	37±3	60±4*
22:6 n-3	79±11	141±10*	60±5	166±16*	79±12	198±17*

* significantly different from Day 1 value determined by ANOVA ($p<0.05$).

Importantly, there were no increases in plasma AA levels observed at any of the dose levels. In fact, in the 100g dose group the concentration of plasma AA as well as that of 18:2 and 18-1 n-9 were significantly decreased most likely due to the competition for acylation sites from other long chain fatty acids like EPA, DHA and DGLA. In the case of 18:1 and 18:2, a significant contribution to the decreased plasma content may have also been caused by the 40% decrease in plasma triglyceride concentrations in the 100g group. Fatty acid concentrations in the placebo group consuming the emulsion containing olive oil were unchanged throughout the study (data not shown).

LTB_4 Biosynthesis. The enhanced tissue enrichment with fatty acids in subjects consuming the oral PUFA emulsion of this invention also had an impact on stimulated LTB_4 biosynthesis in whole blood. As with subjects consuming gelatin capsules, the consumption of 1.5g GLA and 1g EPA per day for 14 days resulted in a significant decrease in the capacity to synthesize LTB_4 when compared to placebo (FIG. 19A and FIG. 19B). In contrast to gelatin capsules, the consumption of much lower doses of GLA (0.75g) and EPA (0.5g) provided as an emulsion also markedly attenuated stimulated blood LTB_4 biosynthesis when compared to placebo (FIG. 23). However, subjects consuming the 100g dose did not show a change in LTB_4 when compared to placebo. This unexpected result may be explained by the lack of a significant increase in plasma DGLA concentrations in this group. Indeed, the greatly enhanced concentrations of tissue EPA and DHA may have prevented the acylation of DGLA into glycerolipids thus dampening the blocking effect of dietary GLA on LTB_4 biosynthesis.

Although alteration of LTB_4 biosynthesis was exemplified herein, other arachidonic acid metabolites may be affected including, without limitation, the arachidonic acid metabolites shown in FIGS. 24 and 25.

Use of the Composition of the Invention for the Management of Asthma:

Asthma is characterized by the stimulus-derived release of mediators such as histamine and leukotrienes from inflammatory cells leading to reversible airway narrowing, mucus secretion and inflammation. Seasonal and perennial allergens are examples of stimuli that induce the activation and degranulation of mast cells leading to mediator release. A variety of therapeutics products such as beta-agonists, topical and systemic steroids, antihistamines, phosphodiestarase-4 inhibitors and leukotriene antagonist have been used to address asthma and allergy symptoms.

Asthmatics synthesize higher level of leukotrienes when compared to their non-asthmatic counterparts (*Pacheco et al., Eur. J. Clin. Invest.* 1992;22(11):732-9; *Radeau et al., Prostaglandins Leukot Essent Fatty Acids* 1990;41(2):131-8; *Shindo et al., Thorax.* 1997;52(12):1024-9; *Cheria-Sammari et al., Clin. Exp. Allergy* 1995;25(8):729-36; *Sampson et al., Br. J. Clin. Pharmacol.* 1992;33(4):423-30) and leukotriene receptor antagonists inhibit these airway effects by blocking leukotriene

binding to the cys-1 leukotriene receptor. Leukotriene inhibitors (receptor antagonists and 5-lipoxygenase blockers) attenuate bronchoconstriction and airway inflammation caused in part by the release of leukotrienes from cells in airways such as mast cells, neutrophils and eosinophils (*Tashkin, Allergy Asthma Proc.* 2001;22(5):311-9). The beneficial effects of leukotriene antagonists on exercise-induced asthma in children (*Pearlman et al., J. Pediatr.* 1999;134(3):273-9; *Leff et al., N Engl. J. Med.* 1998;339(3):147-52) and their sparing effects on oral corticosteroids and β-agonist use in atopic asthmatic children (*Knorr et al., Pediatrics* 2001;108(3):E48) are especially noteworthy. Several recent clinical trials have also suggested a potential therapeutic benefit of leukotriene receptor antagonists and inhibitors in other conditions with an inflammatory component like allergic rhinitis (*Meltzer et al., J. Allergy Clin. Immunol.* 2000;105(5):917-22; *Wilson et al., Clin. Exp. Allergy* 2001;31(4):616-24) and inflammatory bowel disease (*Hawkey et al., Gastroenterology* 1997;112(3):718-24). In view of the critical nature of leukotrienes in human disease, it is important that strategies be developed to normalize elevated leukotriene levels. We and others have reported novel therapeutic strategies to reduce leukotriene levels (*Pullman-Mooar et al., Arthritis Rheum.* 1990;33(10):1526-33; *Ziboh et al., Am. J. Clin. Nutr.* 1992;55(1)39-45; *Barham et al., J.Nutr.* 2000;130(8):1925-31). For example, ex-vivo LTB_4 biosynthesis is markedly blocked in humans supplementing their controlled eucaloric diets with high doses of dietary GLA (3g GLA/day) for a 3-week period (*Johnson et al., J. Nutr.* 1997;127(8)1435-44; *Barham et al., J. Nutr.* 2000;130(8):1925-31). However, until the current study, there was no clear indication of the minimum effective dose of GLA necessary to affect leukotriene biosynthesis. The current study demonstrates that 1.5g of GLA per day provided as gelatin capsules or 0.75 g of GLA provided as an emulsion significantly decreases stimulated LTB_4 biosynthesis within 2 weeks while the daily consumption of 0.75g of GLA in gel capsules (equivalent to 0.375 g of GLA in an emulsion) for up to 3 weeks had no measurable effect on LTB_4 biosynthesis. In previous dietary GLA studies in our laboratory, subjects consumed a controlled diet prepared in the metabolic kitchen of a General Clinical Research Center (*Johnson et al., J. Nutr.* 1997;127(8)1435-44; *Barham et al., J. Nutr.* 2000;130(8):1925-31). The current study reveals that this rigorous dietary control was not necessary to obtain significant decreases in stimulated LTB_4 biosynthesis.

In contrast to several cells and tissues such as the liver, human inflammatory cells such as the neutrophil do not contain the Δ5-desaturase enzyme and thus cannot convert DGLA to AA (*Chilton et al., J. Immunol.* 1996;156(8):2941-7). Our laboratory also recently reported that dietary supplementation with GLA at levels required to reduce leukotrienes results in the potentially deleterious side effect of elevated plasma AA levels. This increase in plasma AA levels is thought to be due to the Δ^5-desaturase-catalyzed conversion of DGLA to AA in the liver (*Johnson et al., J. Nutr.* 1997;127(8):1435-44; *Chilton et al., J. Immunol.* 1996;156(8):2941-7). Increased serum AA concentrations associated with the consumption of dietary AA have been suggested to increase platelet reactivity (*Seyberth et al., Clin. Pharmacol. Ther.* 1975;18(5 Pt 1):521-9) and chronically elevated levels of AA in the plasma could potentially negate the anti-inflammatory benefits of GLA. Our strategy for limiting this increase in plasma AA levels was to co-supplement the diet with the Δ^5-desaturase inhibitor, EPA. EPA prevents the in vitro conversion of DGLA to AA possibly by inhibiting Δ^5-desaturase (*Barham et al., J. Nutr.* 2000;130(8)1925-31). Accordingly, the inclusion of n-3 fatty acids in the diet at the correct GLA to EPA ratios prevents the increase in plasma AA associated with the consumption of GLA (*Barham et al., J. Nutr.* 2000;130(8)1925-31). The current study demonstrated that the inclusion of n-3 PUFA between 0.7g/day to 2.2g/day (0.25g to 1.0g EPA per day) was sufficient to prevent the increase in plasma AA associated with the consumption of 1.5g GLA per day. These initial studies established the ratios of GLA and EPA provided in gelatin capsules effective to reduce leukotriene biosynthesis while preventing increases in plasma AA levels.

These studies also revealed a practical limitation to the utilization of gelatin capsules containing oils to control leukotriene biosynthesis; specifically, seven to nine large gelatin capsules containing nearly 4g of concentrated Borage oil and 3g of concentrated marine oil were required to control AA metabolism. To overcome this obstacle, the liquid PUFA emulsion of the present invention was developed to deliver efficacious quantities of these oils in a single daily dose. Administration of this emulsion resulted in an approximately two-fold greater absorption of these fatty acids as compared to gelatin capsules. In fact, plasma PUFA concentrations achieved in subjects consuming 10g/day of our emulsion (delivering 0.75g GLA and 0.5g EPA per day) were similar to those measured in subjects consuming gelatin capsules

delivering 1.5g GLA and 1g EPA per day. In so far as in humans, this is the first report demonstrating that oil-in-water emulsions are superior for delivery of PUFAs to gelatin capsules.

Utilizing information extracted from the clinical trials, 10g of emulsion containing 0.75g GLA and 0.5g EPA was provided daily to human subjects. The fatty acid emulsion induced a marked decrease in blood LTB_4 levels and the magnitude of the decrease is of potential therapeutic benefit to patients suffering from diseases with an inflammatory component involving leukotrienes such as asthma. For example, it is established that the inhibition of leukotriene biosynthesis by 5-lipoxygenase inhibitors results in an amelioration of clinical symptoms of asthma such as forced expiratory volume in one second (FEV_1) or the frequency of use of rescue inhalers (*Israel et al., Ann. Intern. Med.* 1993;119(11):1059-66. In fact, the inhibition of LTB_4 biosynthesis in whole blood has been used as a pharmacodynamic endpoint in determining the therapeutic dose of 5-LO inhibitors (Israel et al., N. Engl. J. Med. 1990;323(25):1740-4). There was no further inhibition of blood LTB_4 biosynthesis in subjects administered the oral PUFA emulsioin of this invention at doses higher than 10g/day. In fact, LTB_4 levels remained unchanged in the subjects consuming the highest dose of our emulsion (100g/day). Light was shed on this unexpected finding when it was discovered that DGLA was not incorporated into tissues of subjects consuming the 100g/day dose of our emulsion. This observation again suggests that DGLA or a DGLA metabolite plays an important and critical role in the observed inhibition of LT biosynthesis.

Several studies have shown that leukocytes from atopic individuals, including atopic asthmatics, synthesize significantly more leukotrienes than their normal counterparts (*Pacheco et al., Eur. J. Clin. Invest.* 1992;22(11):732-9; *Radeau et al., Prostaglandins Leukot Essent Fatty Acids* 1990;41(2):131-8; *Shindo et al., Thorax.* 1997;52(12):1024-9; *Cheria-Sammari et al., Clin. Exp. Allergy* 1995;25(8):729-36; *Sampson et al., Br. J. Clin. Pharmacol.* 1992;33(4):423-30). It has been established that normalization of leukotriene biosynthesis in asthmatic individuals attenuates the incidence and/or severity of asthma.

A daily consumption of 10g or 20g of the oral PUFA emulsion of the invention for 2 weeks was well tolerated and was not associated with any abnormal clinical laboratory values or any differences in treatment-related adverse events as

compared to placebo. Pharmacokinetic analyses revealed that stable trough levels of GLA and EPA were established by 7 days at all doses of the emulsion form of the dosage unit. Transient increases in liver serum transaminases (SGPT) were observed in 3 individuals consuming ten times the recommended dose of the emulsion (100g per day). Increases in the average serum activity of liver enzymes have been observed previously following the daily consumption of high quantities of marine oils (*Eritsland, Am. J. Clin. Nutr.* 2000;71(1 Suppl):197S-201S; *Schmidt et al., Drug Investig.* 1994;7(4):215-20).

Taken together, the studies reported here have defined parameters critical to safely impacting leukotriene biosynthesis with dietary PUFA in humans. These studies clearly point out that there are unexpected lower and upper ranges of PUFAs and ratios of active fatty acids such as GLA and EPA needed to safely inhibit leukotriene biosynthesis in a chronic leukotriene-dependent disease such as asthma. Our studies point out that there is an important ceiling at which ingested PUFAs will cause an elevation in liver transaminases and it is important to remain below that ceiling when providing PUFAs for the treatment of chronic human diseases. This information can now be used to design therapies for the management of lipid-mediated disorders or conditions including inflammatory diseases, such as, asthma, allergic rhinitis or inflammatory bowel disease, where blocking the overproduction of leukotrienes is known to have an impact on the disease.

Example 13

An Emulsion containing Borage Oil and Marine Oil Suppresses Leukotriene Synthesis in Asthmatics

Tissue Necrosis Factor α (TNFα) and Interleukin 1 β (IL-1 beta), cytokines produced by activated T cells, mast cells, macrophages and eosinophils, have been shown to play a critical role in the pathogenesis of several inflammatory diseases including rheumatoid arthritis and asthma.

TNFα and IL-1 beta have been found in the inflamed joints of a rheumatoid arthritis patient (Fontana et al., Rhumatol Int 1982 2:49-53; DiGiovine F et al., Ann Rheum Dis 1988 47:768-72; Buchan G et al., Clin Exp Immunol 1988 73:449-55) and have been implicated in several biological actions that lead to the pathology of rheumatoid arthritis (RA) including synovial hyperplasia, leukocyte infiltration

(Arend WP et al., Arthitis Rheum 1990 33:305-15) and cartilage degradation (Bevilacqua MP et al., J Clin Invest 1985 76:2003-11). In addition, higher levels of IL-1 beta, TNFα and prostaglandin E_2 (PGE_2) were produced by peripheral blood monocytes from patients with RA when compared to healthy individuals (Mazurov et al., Ter Arkh 1992 64:20-4; Fujii I et al., Ann Rheum Dis 1990 49:497-503; Yamamura M et al., Acta Med Okayama 1990 44:13-20; Bomalaski JS et al., Arthritis Rheum 1986 29:312-8.

Polyunsaturated n-3 oils such as eicosapentaenoic acid (EPA) have been reported to reduce the generation of IL-1 beta and TNFα by peripheral blood monocytes (Endres S et al., 1989 N Engl J Med. 320:265-271). These oils have been used in clinical trials of patients with RA with positive results in suppressing inflammation (Lau et al Br. J Rheum. 1993 32:982-989; Kremer JM et al., Ann. Intern Med. 1987 106:4997-504; Skoldskam et al., Arthritis Rheum. 1990 33:810-820).

In another placebo controlled trial, significant reductions in IL-1 beta, TNF alpha and IL-6 as well as PGE_2 were reported in RA patients consuming blackcurrant oil rich in GLA (Watson J et al., 1993 32:1055-8). Dietary Borage oil containing GLA was also tested in RA patients with similar positive results reported on the reduction of tender joint pain and with no observed effect in placebo (Leventhal et al., Ann Intern Med 1993 119:867-73; Leventhal et al., Br J Rheum. 1994 33:847-52).

As mentioned in the previous examples, leukotrienes play a significant role in clinical asthma. The emulsion of the present example is a mixture of natural dietary fats including gamma linolenic acie (GLA) and eicospentaenoic acid (EPA) derived from borage oil and marine oils, respectively. The present example is directed to an analysis of the ability of this emulsion to suppresss stimulated LT and cytokine production in whole blood from asthmatics in the absence of dietary modification .

Methods for Example 13

An analysis was performed on 23 atopic subjects with mild to moderate asthma controllable solely with inhaled beta-agonists or theophylline, without inhaled or systemic steroids. The data represent the results of a randomized, double blind, parallel group trial wherein patients received placebo (n=5), low-dose (n=9), or high-dose (n=9) emulsion containing borage oil and marine oil. Prior to study completion 5 subjects withdrew and 3 subjects were excluded because of non-compliance.

Stimulated ex-vivo LTB4 and TNF-alpha were measure at baseline and after 4 weeks of supplementation. LTB4 was measured as described in the previous example. ELISA was utilized to quantitate TNF-alpha levels in plasma obtained from whole blood that had been stimulated ex-vivo with lipopolysaccharide.

Results

Two intermediate dose emulsions containing borage oil and marine oil suppressed biosynthesis of LTB4 (31.1±4.1% and 19.9±3.9% reduction, respectively) compared to placebo (6.1±7.1% increase), $p<0.05$. Similarly, both intermediate emulsion doses suppressed TNF-alpha synthesis (31±5.5% and 4.4±10% reduction respectively) compared to placebo (41±11.3% increase), $p<0.05$. See Figure 26.

We conclude from these studies that the emulsion described in the present example, a once per day medical food, represents a new approach for modifying LTs thereby providing yet another modality for the treatment of asthma.

While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

We claim:

1. A composition consisting essentially of a leukotriene inhibitor in an amount from about 0.02 grams to about 3 grams, and a Δ^5desaturase inhibitor in an amount effective to prevent an increase in arachidonic acid metabolites, said composition optionally comprising an inhibitor of arachidonic acid metabolism.

2. The composition of claim 1, wherein said Δ^5 desaturase inhibitor is selected from the group consisting of eicosapentanoic acid, sesamin, episesamin, sesaminol, sesamolin, curcumin, alpha-linolenic acid, heneicosapentaenoic acid, docosahexaenoic acid, alkyl gallate, propyl gallate or *p*-isopentoxyaniline.

3. The composition of claim 1 wherein said Δ^5 desaturase inhibitor is eicosapenataenoic acid (EPA).

4. The composition of claim 3, wherein said EPA is present in an amount from about 0.02 grams to about 3 grams.

5. The composition of claim 1, wherein said leukotriene inhibitor is selected from the group consisting of GLA and DGLA.

6. The composition of claim 5, wherein said leukotriene inhibitor is GLA.

7. The composition of claim 6 wherein said GLA is present in an amount from about .75 to about 3 grams.

8. The composition of claim 5, wherein said leukotriene inhibitor is DGLA.

9. The composition of claim 5, optionally further comprising a platelet activating factor inhibitor.

10. The composition of claim 7 in dosage unit form, said dosage unit providing a daily dose of said EPA and said GLA.

11. The composition of claim 8 in dosage unit form, said dosage unit providing a daily dose of said EPA and said DGLA .

12. The composition of claim 1, optionally further comprising at least one ingredient selected from the group consisting of a flavoring agent, a sweetening agent, a coloring agent or a preservative.

13. The composition of claim 1, wherein said inhibitor of arachidonic acid metabolism is a competitive inhibitor.

14. An emulsion consisting essentially of a leukotriene inhibitor in an amount from about 0.02 grams to about 3 grams, a Δ^5desaturase inhibitor in an amount effective to prevent an increase in arachidonic acid metabolites, at least one emulsifying agent or emulsion stabilizer, and water, said emulsion optionally further comprising an inhibitor of arachidonic acid metabolism.

15. The emulsion of claim 14, wherein said Δ^5 desaturase inhibitor is selected from the group consisting of eicosapentanoic acid, sesamin, episesamin, sesaminol, sesamolin, curcumin, alpha-linolenic acid, heneicosapentaenoic acid, docosahexaenoic acid, alkyl gallate, propyl gallate or *p*-isopentoxyaniline.

16. The emulsion of claim 14 wherein said Δ^5 desaturase inhibitor is eicosapenataenoic acid (EPA).

17. The emulsion of claim 16, wherein said EPA is present in an amount from about 0.02 grams to about 3 grams.

18. The emulsion of claim 14, wherein said leukotriene inhibitor is selected from the group consisting of GLA and DGLA.

19. The emulsion of claim 18, wherein said leukotriene inhibitor is GLA.

20. The emulsion of claim 19, wherein said GLA is present in an amount

from about 0.75 to about 3 grams.

21. The emulsion of claim 19, wherein said leukotriene inhibitor is DGLA.

22. The emulsion of claim 19, optionally further comprising a platelet activating factor inhibitor.

23. The emulsion of claim 19 in dosage unit form, said dosage unit providing a daily dose of EPA and said GLA.

24. The emulsion of claim 19 in dosage unit form, said dosage unit providing a daily dose of EPA and said DGLA

25. The emulsion of claim 24, optionally further comprising at least one ingredient selected from the group consisting of a flavoring agent, a sweetening agent, a coloring agent or a preservative.

26. The emulsion of claim 16, wherein said inhibitor of arachidonic acid metabolism is a competitive inhibitor.

27. The emulsion of claim 14, wherein said at least one emulsifying agent or emulsion stabilizer is selected from the group consisting of phospholipids, lecithin, xanthan gum, guar gum, pectin, carob seed gum (locust-bean gum), tragacanth gum, methylcellulose, alginates, carrageenan, starch, modified starch, carboxymethylcellulose, gum Arabic, and gelatin.

28. The emulsion of claim 20, wherein said at least one emulsifying agent or emulsion stabilizer is selected from the group consisting of phospholipids, lecithin, xanthan gum, guar gum, pectin, carob seed gum (locust-bean gum), tragacanth gum, methylcellulose, alginates, carrageenan, starch, modified starch, carboxymethylcellulose, gum Arabic, and gelatin.

29. The emulsion of claim 14, the bioavailability of the emulsified GLA and

EPA being greater than the bioavailability of GLA and EPA administered in gel capsule form.

30. A method of treating a lipid-mediated disorder or a disorder having an arachidonic acid metabolite component in a patient in need of such treatment by administering to said patient an effective amount of the composition of claim 1.

31. The method of claim 30, wherein said disorder is at least one of asthma, allergic rhinitis, allergic rhinoconjunctivitis, psoriasis, acute myocardial infarction, glomerulonephritis, Crohn's disease, inflammatory bowel disease (IBD), arthritis, breast cancer, colon cancer, prostate cancer, squamous cell carcinoma, intestinal cancer, ovarian cancer, uterine cancer , testicular cancer, autoimmune diseases, systemic Lupus erythematosus, schizophrenia, depression, IgA nephropathy, sepsis, toxic shock, organ failure, organ transplant, coronary angioplasty, cystic fibrosis, atherosclerosis, atopic dermatitis, menstrual discomfort, cyclic breast pain, premature labor, early parturition, gout, venous leg ulcers, chronic urticaria, thyroiditis, primary dysmenorrhea, endometriosis, Lyme disease, muscle wasting, ankylosing spondylitis, carpal tunnel syndrome, childhood or juvenile arthritis, chronic back injury, fibromyalgia, gout, infectious arthritis, osteoarthritis, osteoporosis, Pagets's Disease, polymyalgia rheumatica, polymyositis , dermatomyositis, pseudogout, psoriatic arthritis, Raynaud's Syndrome, reactive arthritis, Reiter's Syndrome, repetitive stress injury, rheumatoid arthritis, scleroderma, sickle cell anemia, eczyma, and Sjögrens Syndrome.

32. The method of claim 30, wherein said lipid-mediated disorder is asthma, arthritis, Crohn's disease, inflammatory bowel disease, breast cancer, colon cancer and prostate cancer, squamous cell carcinoma, premature labor, early parturition, and muscle wasting.

33. The method of claim 30, wherein said disorder is asthma.

34. The method of claim 30, wherein administration of said composition alters synthesis of at least one arachidonic acid metabolite.

35. The method of claim 30, wherein said arachidonic acid metabolite is selected from the group consisting of at least one of leukotriene , prostaglandin, hydroxy fatty acid and lipoxin.

36. The method of claim 30, wherein said arachidonic acid metabolite is selected from the group consisting of LTA4; LTB4; LTC4; LTD4; LTE4; 5(S)-HETE; lipoxin A4; lipoxin B4; 15-epi-lipoxinA4; 15-epi-lipoxin A5; PGH2; PGE2; PGD2; PGI2; 6-keto-PGF2α; TXA2 and TXB2.

37. The method of claim 35, wherein said arachidonic acid metabolite is LTB_4.

38. A method of treating a lipid-mediated disorder or a disorder having an arachidonic acid metabolite component in a patient in need of such treatment by administering to said patient an effective amount of the emulsion of claim 14.

39. The method of claim 38, wherein said disorder is at least one of asthma, allergic rhinitis, allergic rhinoconjunctivitis, psoriasis, acute myocardial infarction, glomerulonephritis, Crohn's disease, inflammatory bowel disease, arthritis, breast cancer, colon cancer, prostate cancer, squamous cell carcinoma, intestinal cancer, ovarian cancer, uterine cancer , testicular cancer, autoimmune diseases, systemic Lupus erythematosus, schizophrenia, depression, IgA nephropathy, sepsis, toxic shock, organ failure, organ transplant, coronary angioplasty, cystic fibrosis, atherosclerosis, atopic dermatitis, menstrual discomfort, cyclic breast pain, premature labor, early parturition, gout, venous leg ulcers, chronic urticaria, thyroiditis, primary dysmenorrhea, endometriosis, Lyme disease, muscle wasting, ankylosing spondylitis, carpal tunnel syndrome, childhood or juvenile arthritis, chronic back injury, fibromyalgia, gout, infectious arthritis, osteoarthritis, osteoporosis, Pagets's Disease, polymyalgia rheumatica, polymyositis , dermatomyositis, pseudogout, psoriatic arthritis, Raynaud's Syndrome, reactive arthritis, Reiter's Syndrome, repetitive stress injury, rheumatoid arthritis, scleroderma, sickle cell anemia, eczeyma, and Sjögrens Syndrome.

40. The method of claim 38, wherein said lipid-mediated disorder is asthma, arthritis, Crohn's disease, inflammatory bowel disease, breast cancer, colon cancer and prostate cancer, squamous cell carcinoma, premature labor, early parturition, and muscle wasting.

41. The method of claim 38, wherein said disorder is asthma.

42. The method of claim 38, wherein administration of said composition alters synthesis of at least one arachidonic acid metabolite.

43. The method of claim 42, wherein said at least one arachidonic acid metabolite is selected from the group consisting of at least one of leukotriene, prostaglandin, hydroxy fatty acid, and lipoxin.

44. The method of claim 42, wherein said arachidonic acid metabolite is selected from the group consisting of LTA4; LTB4; LTC4; LTD4; LTE4; 5(S)-HETE; lipoxin A4; lipoxin B4; 15-epi-lipoxinA4; 15-epi-lipoxin A5; PGH2; PGE2; PGD2; PGI2; 6-keto-PGF2α; TXA2 and TXB2.

45. The method of claim 43, wherein said at least one arachidonic acid metabolite comprises at least one leukotriene.

46. The method of claim 45, wherein said leukotriene is LTB_4.

47. A method for treating a cytokine-mediated disorder in a patient in need of such treatment comprising administration of an effective amount of at least one of GLA or DGLA and a Δ^5 desaturase inhibitor in an amount effective to inhibit synthesis of at least one cytokine, leukotriene, and prostaglandin.

48. The method of claim 47 wherein said Δ^5 desaturase inhibitor is selected from the group consisting of eicosapentaenoic acid, sesamin, episesamin, sesaminol, sesamolin, curcumin, alpha-linolenic acid, heneicosapentaenoic acid, docosahexaenoic acid, alkyl gallate, propyl gallate, or *p*-isopentoxyaniline.

49. The method of claim 48, wherein said Δ^5 desaturase inhibit is EPA.

50. The method of claim 47, wherein said GLA is present in about 0.75 to about 3 grams.

51. A method as claimed in claim 47, wherein said cytokine-mediated disorder is selected from the group consisting of asthma, arthritis, allergic rhinoconjunctivitis, psoriasis, Crohn's disease, inflammatory bowel disease, autoimmune diseases, systemic Lupus erythematosus, IgA nephropathy, sepsis, toxic shock, organ failure, organ transplant, cystic fibrosis, atherosclerosis, atopic dermatitis, eczema, gout, chronic urticaria, thyroiditis, endometriosis, Lyme disease, muscle wasting, ankylosing spondylitis, carpal tunnel syndrome, childhood or juvenile arthritis, fibromyalgia, infectious arthritis, osteoarthritis, osteoporosis, Paget's Disease, polymyalgia rheumatica, polymyositis , dermatomyositis, pseudogout, psoriatic arthritis, Raynaud's Syndrome, reactive arthritis, Reiter's Syndrome, repetitive stress injury, rheumatoid arthritis, scleroderma and Sjögrens Syndrome.

52. The method of claim 51, wherein said cytokine is selected from the group consisting of TNF-β, TNFα and IL-1beta, Il-6, Il-5, and IL-4.

53. The method of claim 52, wherein said cytokine is TNFα.

54. The method of claim 53, wherein said cytokine-mediated disorder is asthma, said cytokine is TNFα and said agent is suspended in a formulation suitable for inhalation.

55. The method of claim 53, wherein said cytokine-mediated disorder is asthma, said cytokine is TNFα and said agent is suspended in a formulation suitable for oral administration.

56. A medical food consisting essentially of a leukotriene inhibitor in an amount from about 0.02 grams to about 3 grams, and a Δ^5desaturase inhibitor in an

amount effective to prevent an increase in arachidonic acid metabolites, said composition optionally comprising an inhibitor of arachidonic acid metabolism.

57. The medical food of claim 56 wherein said Δ^5 desaturase inhibitor is eicosapenataenoic acid (EPA).

58. The medical food of claim 57, wherein said EPA is present in an amount from about 0.02 grams to about 3 grams.

59. The medical food of claim 56, wherein said leukotriene inhibitor is selected from the group consisting of GLA, DGLA, 15-hydroxyeicosatrienoic acid (15-HETRE), 15-hydroxyeicosatetraenoic acid (15-HETE) and 15-hydroxyeicosapentaenoic acid (15-HEPE) .

60. The medical food of claim 56, wherein said leukotriene inhibitor is GLA.

61. The medical food of claim 56, wherein said GLA is present in an amount from about .75 to about 3 grams.

62. The medical food of claim 56, wherein said leukotriene inhibitor is DGLA.

63. The medical food of claim 56, optionally further comprising a platelet activating factor inhibitor.

64. The medical food of claim 57 in dosage unit form, said dosage unit providing a daily dose of said EPA and said leukotriene inhibitor.

65. The composition of claim 57 in dosage unit form, said dosage unit providing a daily dose of said EPA and GLA.

66. A method of treating a lipid-mediated disorder having an arachidonic acid metabolite component in a patient in need of such treatment by administering to said

patient an effective amount of the medical food of claim 56.

67. The method of claim 66, wherein said disorder is at least one of asthma, allergic rhinitis, allergic rhinoconjunctivitis, psoriasis, acute myocardial infarction, glomerulonephritis, Crohn's disease, inflammatory bowel disease, arthritis, breast cancer, colon cancer, prostate cancer, squamous cell carcinoma, intestinal cancer, ovarian cancer, uterine cancer , testicular cancer, autoimmune diseases, systemic Lupus erythematosus, schizophrenia, depression, IgA nephropathy, sepsis, toxic shock, organ failure, organ transplant, coronary angioplasty, cystic fibrosis, atherosclerosis, atopic dermatitis, menstrual discomfort, cyclic breast pain, premature labor, early parturition, gout, venous leg ulcers, chronic urticaria, thyroiditis, primary dysmenorrhea, endometriosis, Lyme disease, muscle wasting, ankylosing spondylitis, carpal tunnel syndrome, childhood or juvenile arthritis, chronic back injury, fibromyalgia, gout, infectious arthritis, osteoarthritis, osteoporosis, Pagets's Disease, polymyalgia rheumatica, polymyositis , dermatomyositis, pseudogout, psoriatic arthritis, Raynaud's Syndrome, reactive arthritis, Reiter's Syndrome, repetitive stress injury, rheumatoid arthritis, scleroderma, sickle cell anemia, eczeyma, and Sjögrens Syndrome.

68. The method of claim 66, wherein said lipid-mediated disorder is asthma, arthritis, Crohn's disease, inflammatory bowel disease, breast cancer, colon cancer and prostate cancer, squamous cell carcinoma, premature labor, early parturition, and muscle wasting.

69. The method of claim 66, wherein said disorder is asthma.

70. The method of claim 66, wherein administration of said agent alters synthesis of at least one arachidonic acid metabolite.

71. The method of claim 70, wherein said at least one arachidonic acid metabolite is selected from the group consisting of at least one of leukotriene, prostaglandin, hydroxy fatty acid, and lipoxin.

72. The method of claim 71, wherein said arachidonic acid metabolite is selected from the group consisting of LTA4; LTB4; LTC4; LTD4; LTE4; 5(S)-HETE; lipoxin A4; lipoxin B4; 15-epi-lipoxinA4; 15-epi-lipoxin A5; PGH2; PGE2; PGD2; PGI2; 6-keto-PGF2α; TXA2 and TXB2.

73. The method of claim 66, wherein said at least one arachidonic acid metabolite comprises at least one leukotriene.

74. The method of claim 73, wherein said leukotriene is LTB_4.

75. A method for the dietary management of asthma, resulting from the inflammatory effect of leukotrienes produced as a by-product of fatty acid metabolism, in a patient in need of said dietary, the method comprising orally administering to said patient having an asthma related dietary deficiency, a medical food consisting essentially of an amount of gamma-linolenic acid (GLA) which is effective to inhibit the inflammatory effect of said leukotrienes.

76. The method of claim 75, wherein the amount of GLA in said medical food is from about 0.75 to about 3.0 grams.

77. The method of claim76, wherein said medical food further includes eicosapentanoicacid (EPA) in an amount from about 0.02g to about 3 grams.

78. The method of claim 77 wherein said medical food further includes water in an amount sufficient to form an emulsion with said GLA and EPA and at least one emulsifying agent or emulsion stabilizer.

79. The method of claim 77, wherein said medical food further includes an inhibitor of arachidonic acid metabolism.

80. The method of claim 77, wherein said medical food further includes at least one ingredient selected from the group consisting of a flavoring agent, a sweetening agent, a coloring agent or a preservative.

81. The method of claim 77, wherein said medical food further includes any macro nutrient selected from the group consisting of protein, carbohydrate and fat.

82. The method of claim 177 wherein said medical food further includes at least one vitamin.

83. A method for the dietary management of asthma, resulting from the inflammatory effect of leukotrienes produced as a by-product of fatty acid metabolism, in a patient in need of said dietary, the method comprising orally administering to said patient having an asthma related dietary deficiency, a medical food consisting essentially of an amount of DGLA which is effective to inhibit the inflammatory effect of said leukotrienes.

84. A composition for treating asthma in an adult consisting essentially of about 0.75 g of GLA and about 0.43 g of EPA.

85. A composition for treating asthma in a pediatric patient consisting essentially of about 0.225 g to about 0.3 g of GLA and about 0.15 to about 0.2 g of EPA.

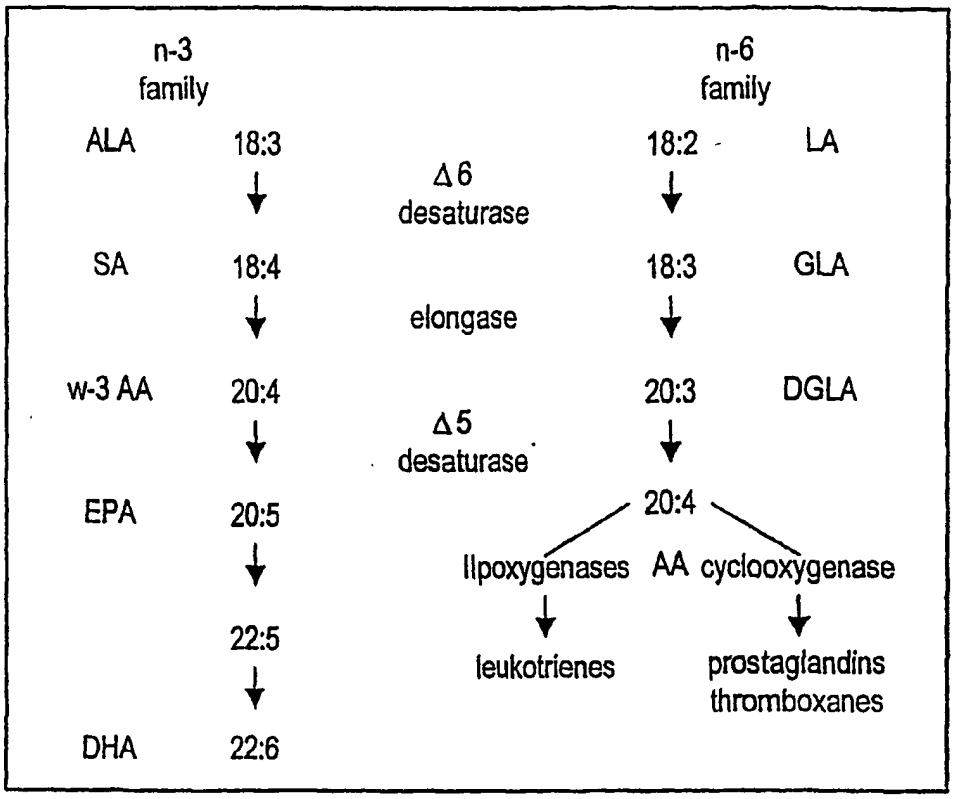
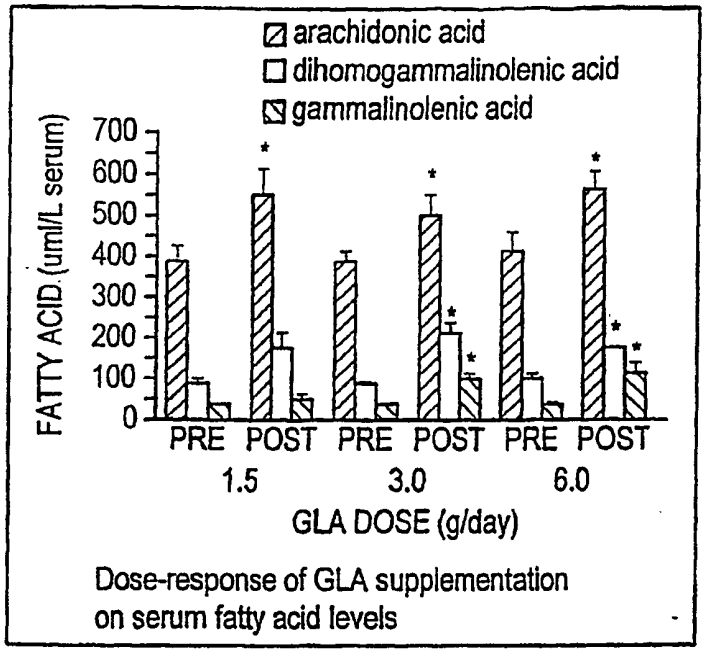
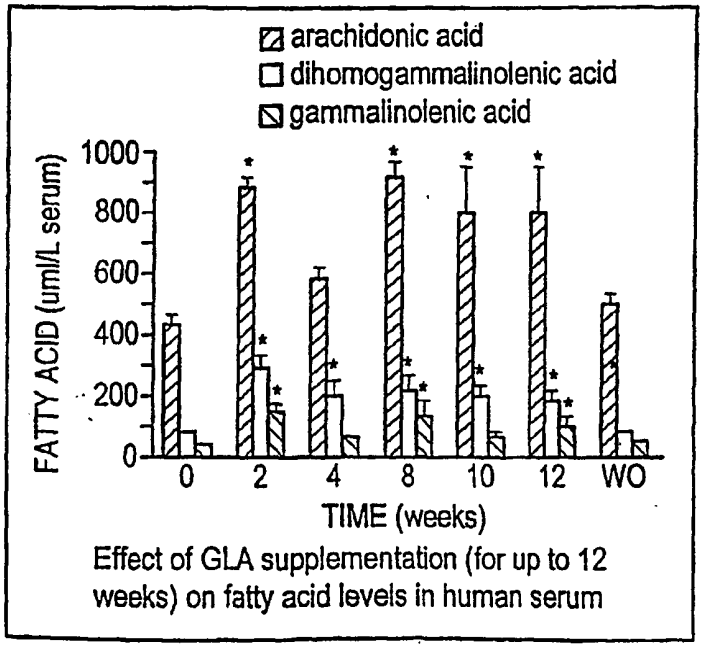

n-3
family
n-6
family
ALA
18:3
Δ6
desaturase
18:2
LA
SA
18:4
elongase
18:3
GLA
w-3 AA
20:4
Δ5
desaturase
20:3
DGLA
EPA
20:5
20:4
lipoxygenases
AA
cyclooxygenase
22:5
leukotrienes
prostaglandins
thromboxanes
DHA
22:6

FIG. 1



Dose-response of GLA supplementation on serum fatty acid levels

FIG. 2



Effect of GLA supplementation (for up to 12 weeks) on fatty acid levels in human serum

FIG. 3

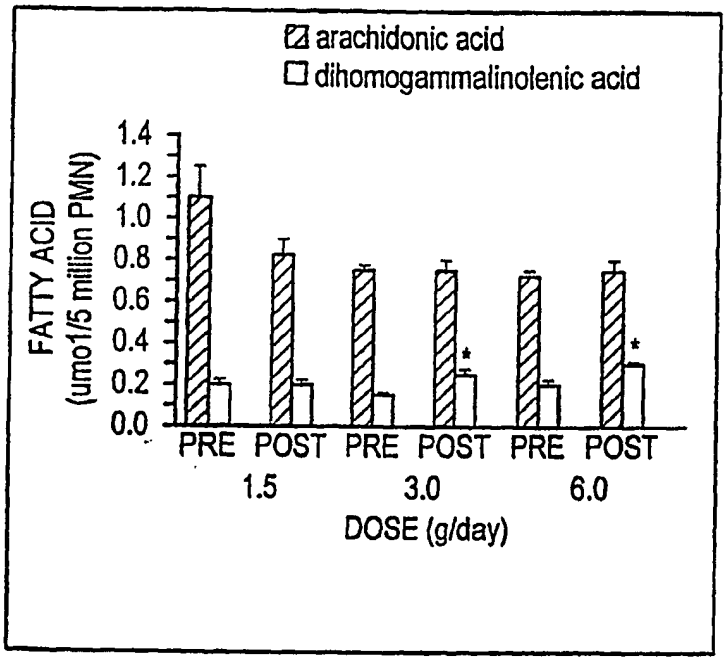

arachidonic acid
dihomogammalinolenic acid
FATTY ACID (umo1/5 million PMN)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
PRE POST PRE POST PRE POST
1.5 3.0 6.0
DOSE (g/day)

FIG. 4

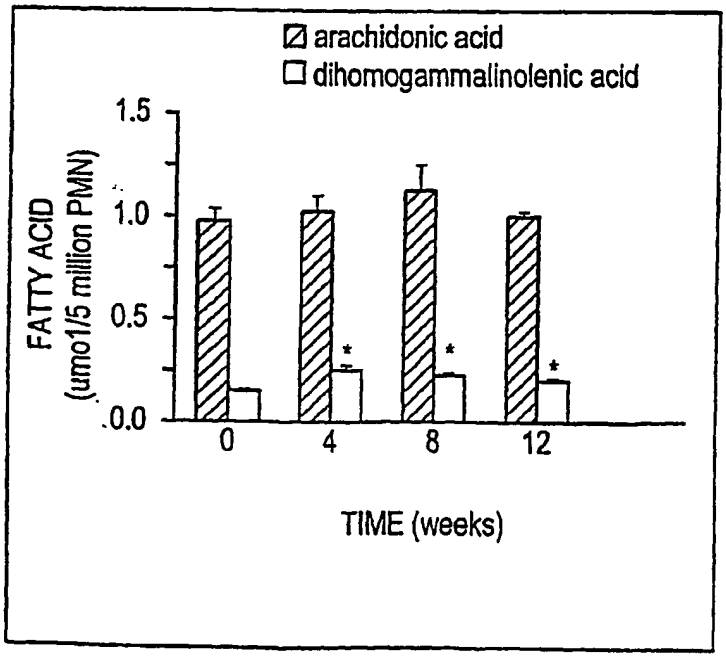

arachidonic acid
dihomogammalinolenic acid
FATTY ACID (umo1/5 million PMN)
1.5
1.0
0.5
0.0
0 4 8 12
TIME (weeks)

FIG. 5

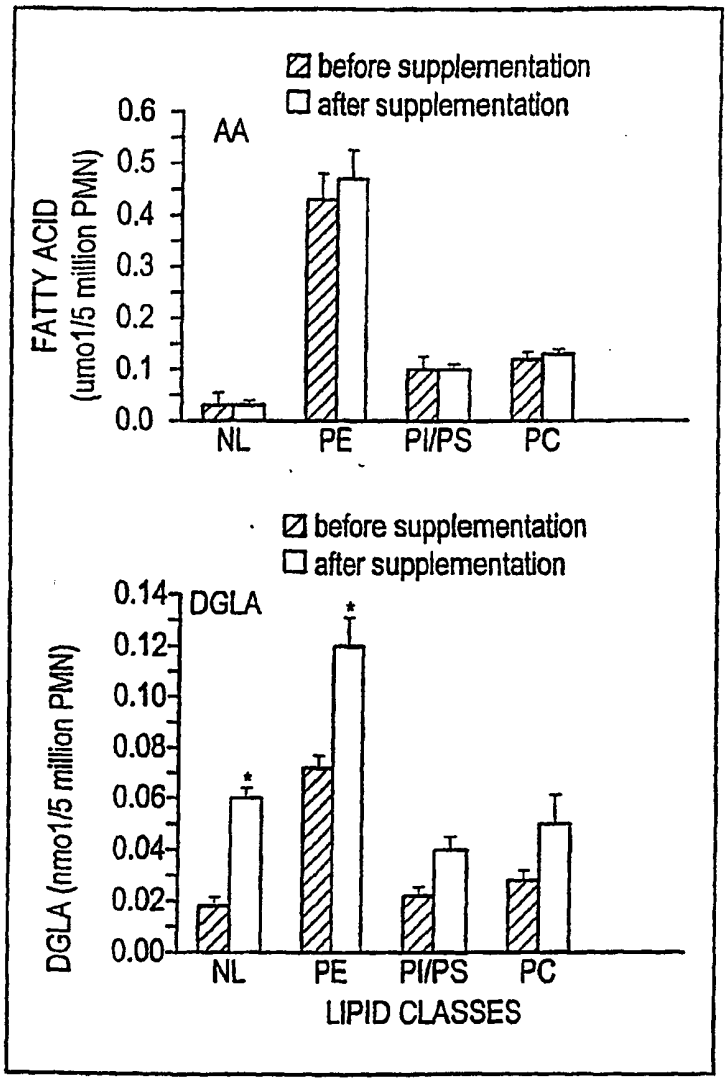

before supplementation
after supplementation
AA
FATTY ACID (umo1/5 million PMN)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
NL
PE
PI/PS
PC
before supplementation
after supplementation
DGLA
DGLA (nmo1/5 million PMN)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
*
*
NL
PE
PI/PS
PC
LIPID CLASSES

FIG. 6A

FIG. 6B

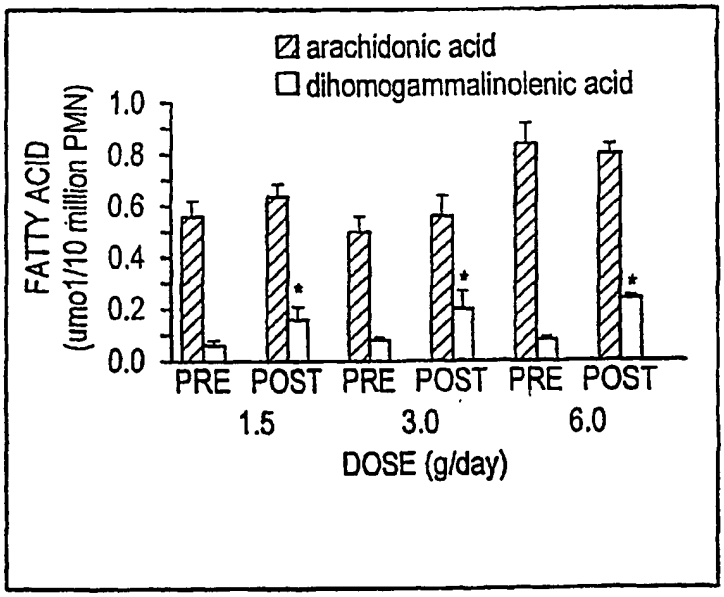

arachidonic acid
dihomogammalinolenic acid
FATTY ACID
(umo1/10 million PMN)
1.0
0.8
0.6
0.4
0.2
0.0
PRE POST PRE POST PRE POST
1.5 3.0 6.0
DOSE (g/day)

FIG. 7

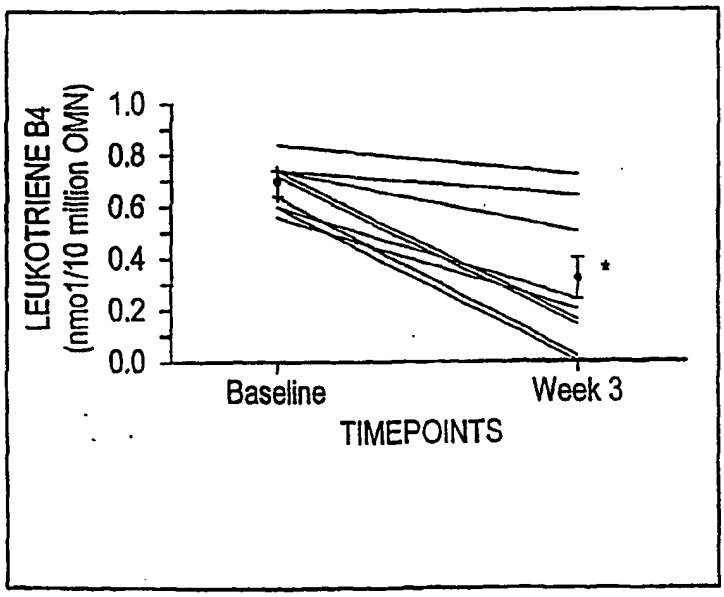

LEUKOTRIENE B4
(nmo1/10 million OMN)
1.0
0.8
0.6
0.4
0.2
0.0
Baseline
Week 3
TIMEPOINTS

FIG. 8

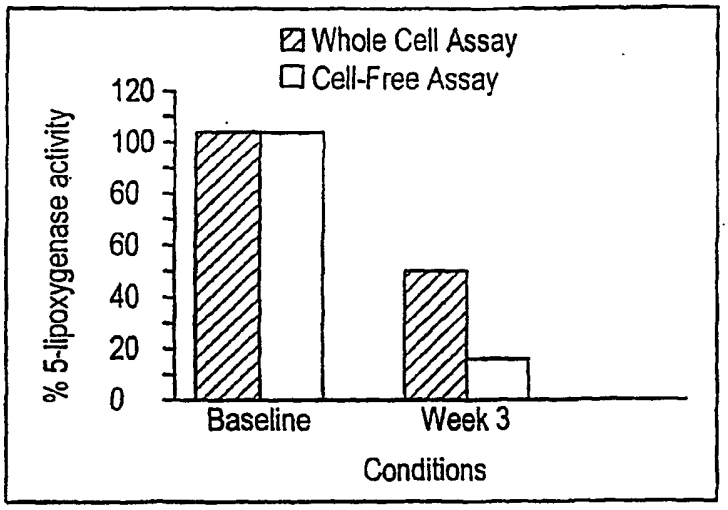

Whole Cell Assay
Cell-Free Assay
% 5-lipoxygenase activity
120
100
60
60
40
20
0
Baseline
Week 3
Conditions

FIG. 9

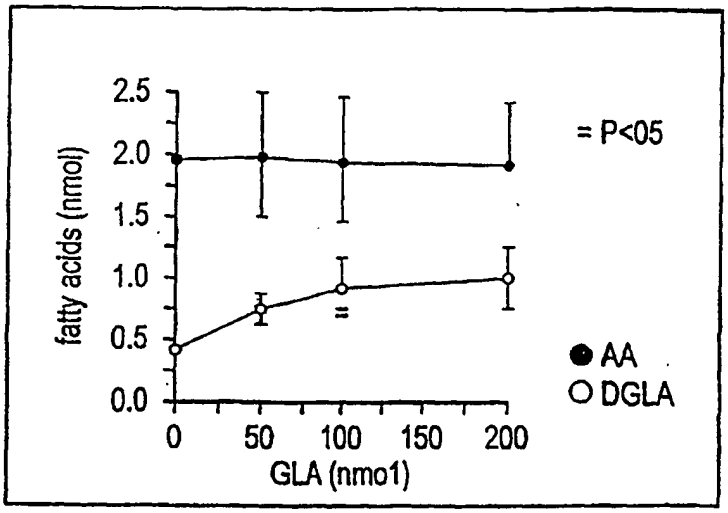

2.5
2.0
1.5
1.0
0.5
0.0
fatty acids (nmol)
0
50
100
150
200
GLA (nmo1)
= P<05
AA
DGLA

FIG. 10

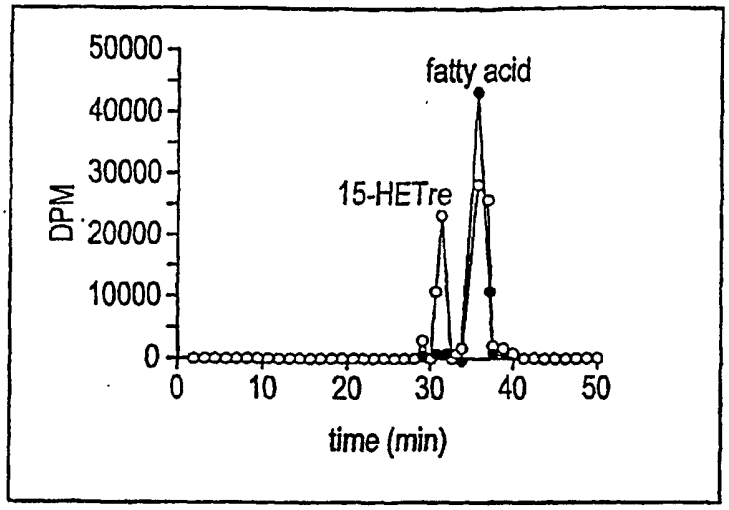

50000
40000
30000
20000
10000
0
DPM
fatty acid
15-HETre
0
10
20
30
40
50
time (min)

FIG. 11

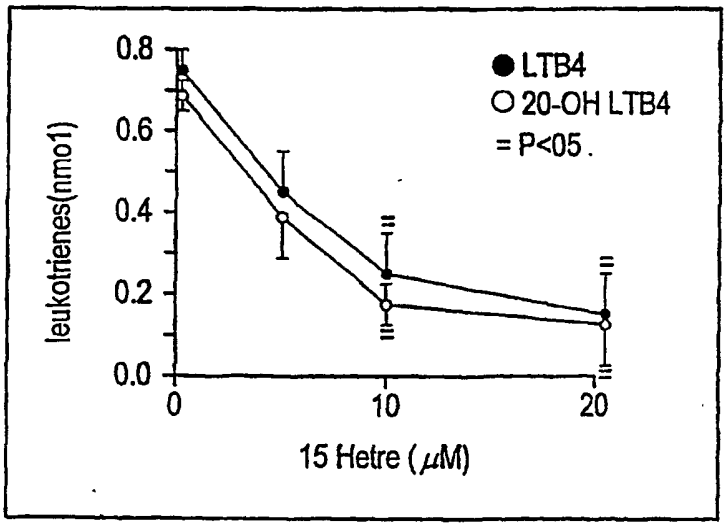

0.8
0.6
0.4
0.2
0.0
leukotrienes(nmol)
LTB4
20-OH LTB4
= P<05.
0
10
20
15 Hetre (μM)

FIG. 12

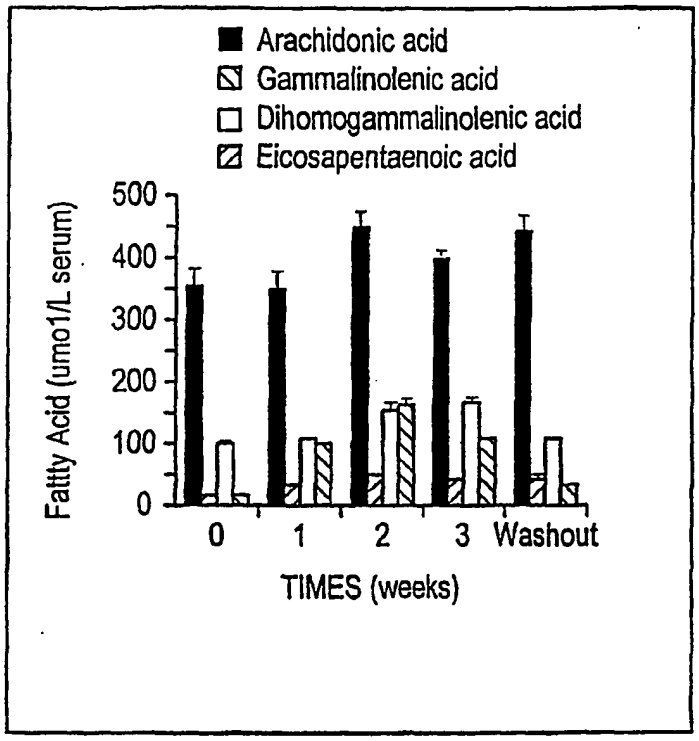

Arachidonic acid
Gammalinolenic acid
Dihomogammalinolenic acid
Eicosapentaenoic acid
Fatty Acid (umo1/L serum)
500
400
300
200
100
0
0
1
2
3
Washout
TIMES (weeks)

FIG. 13

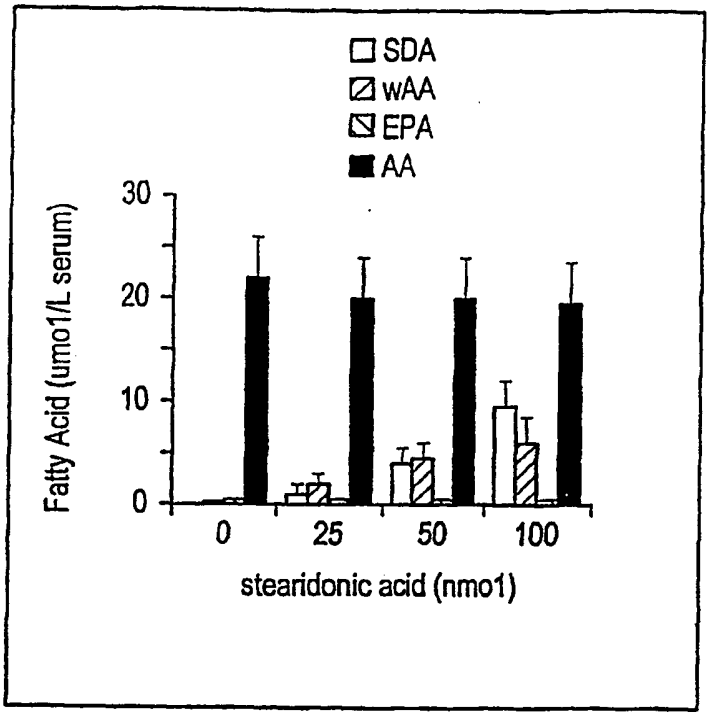

SDA
wAA
EPA
AA
30
20
10
0
Fatty Acid (umo1/L serum)
0
25
50
100
stearidonic acid (nmo1)

FIG. 14

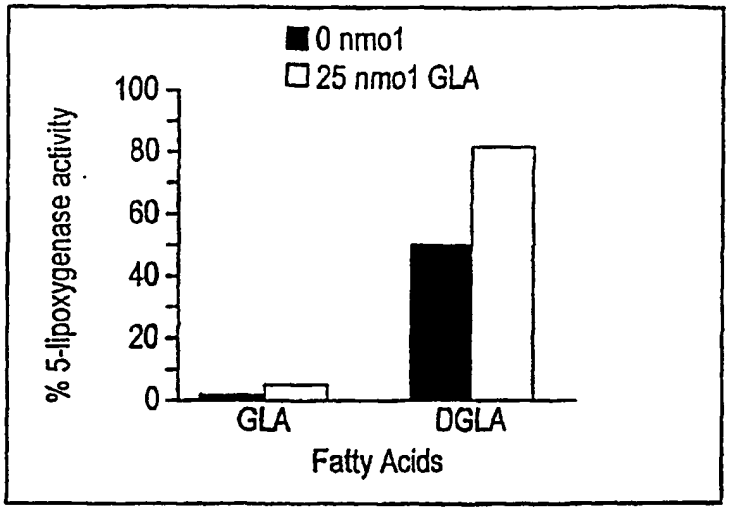

0 nmol
25 nmol GLA
% 5-lipoxygenase activity
100
80
60
40
20
0
GLA
DGLA
Fatty Acids

FIG. 15

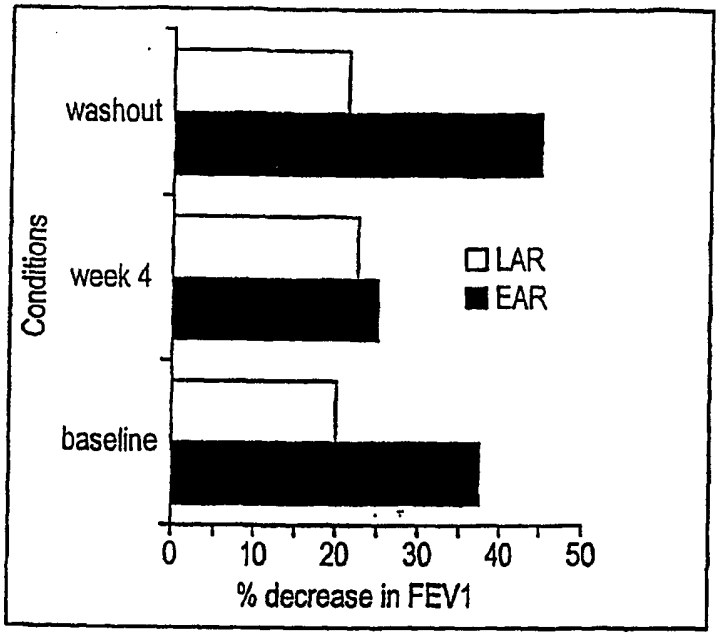

washout
week 4
baseline
Conditions
LAR
EAR
0
10
20
30
40
50
% decrease in FEV1

FIG. 16

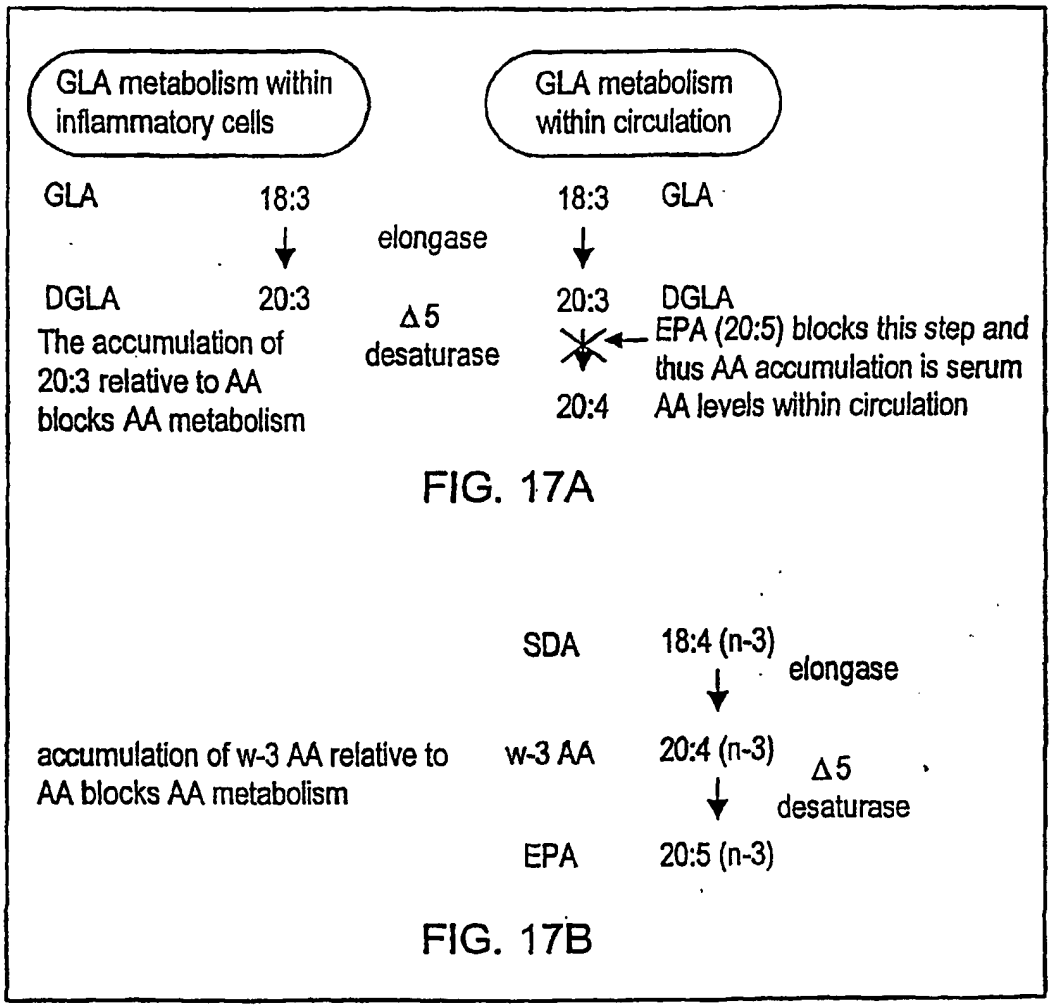


FIG. 17A

FIG. 17B

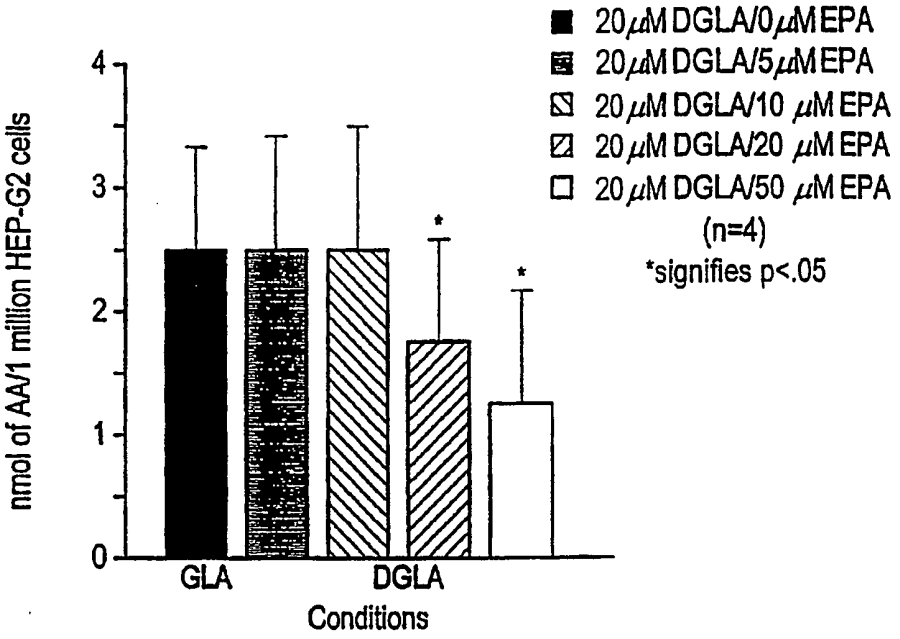


FIG. 18A

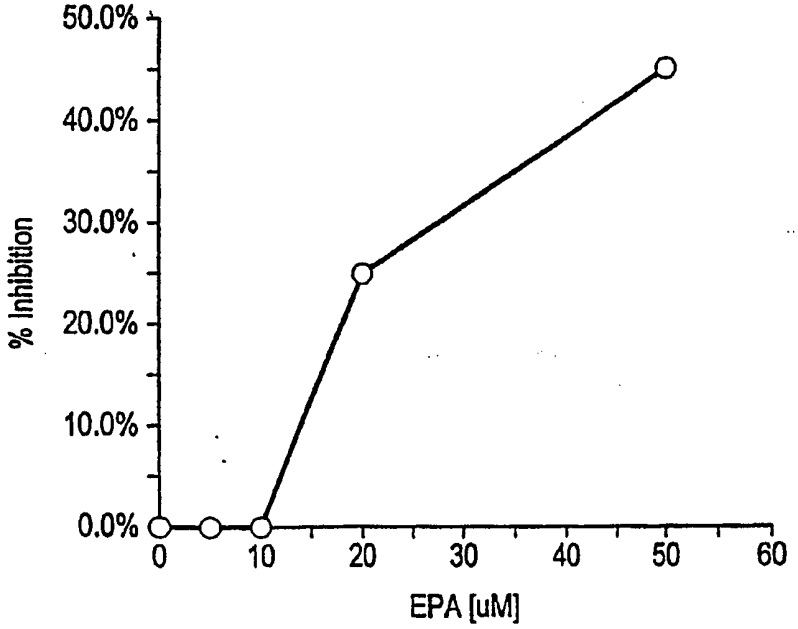


FIG. 18B

FIG. 19A

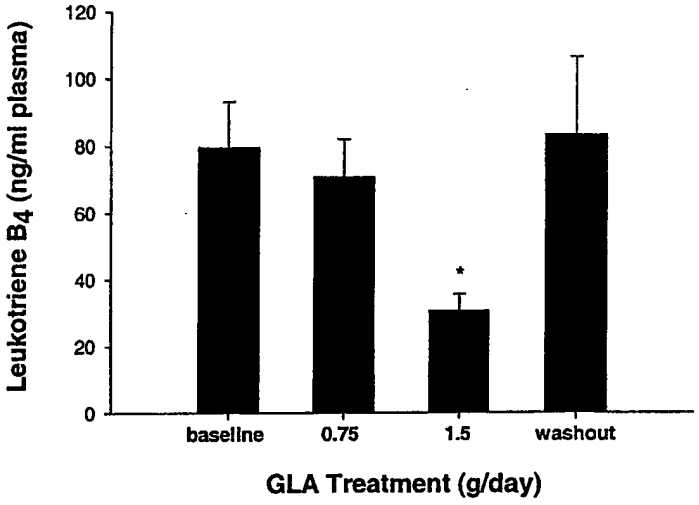


FIG. 19B

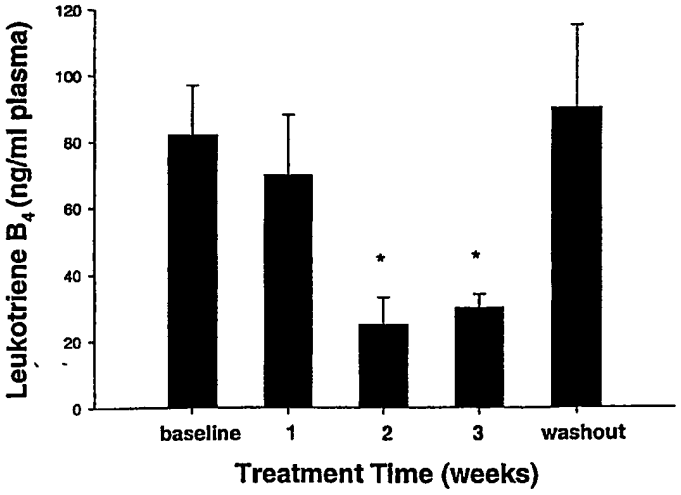


FIG. 20

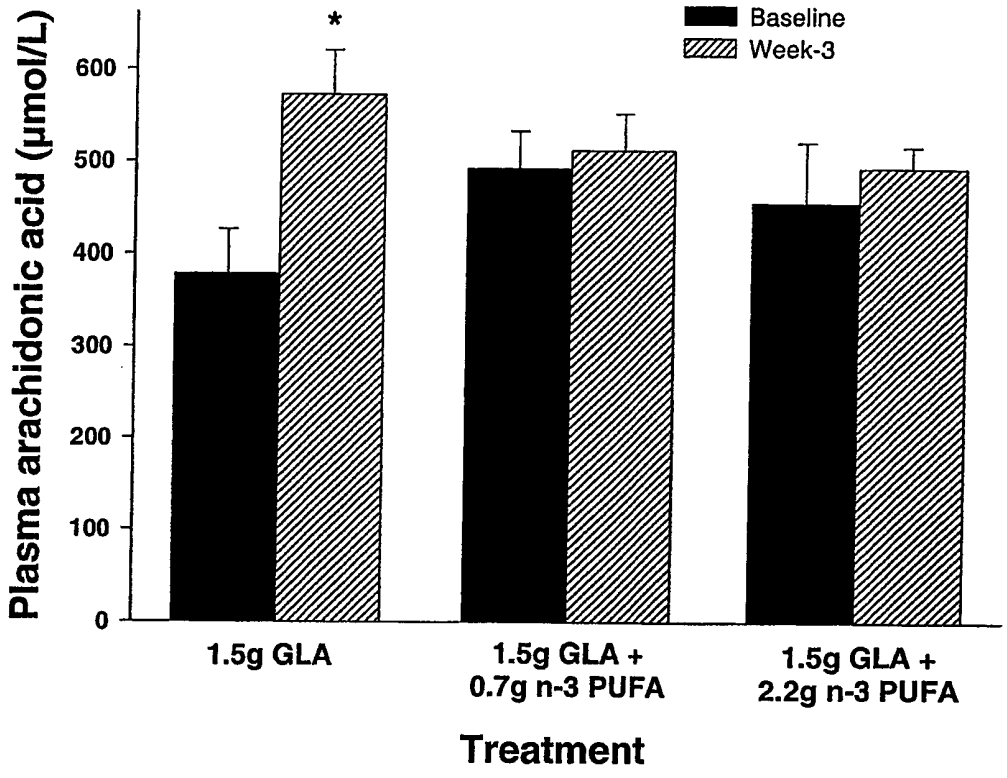

Plasma arachidonic acid (µmol/L)
600
500
400
300
200
100
0
*
Baseline
Week-3
1.5g GLA
1.5g GLA + 0.7g n-3 PUFA
1.5g GLA + 2.2g n-3 PUFA
Treatment

FIG. 21

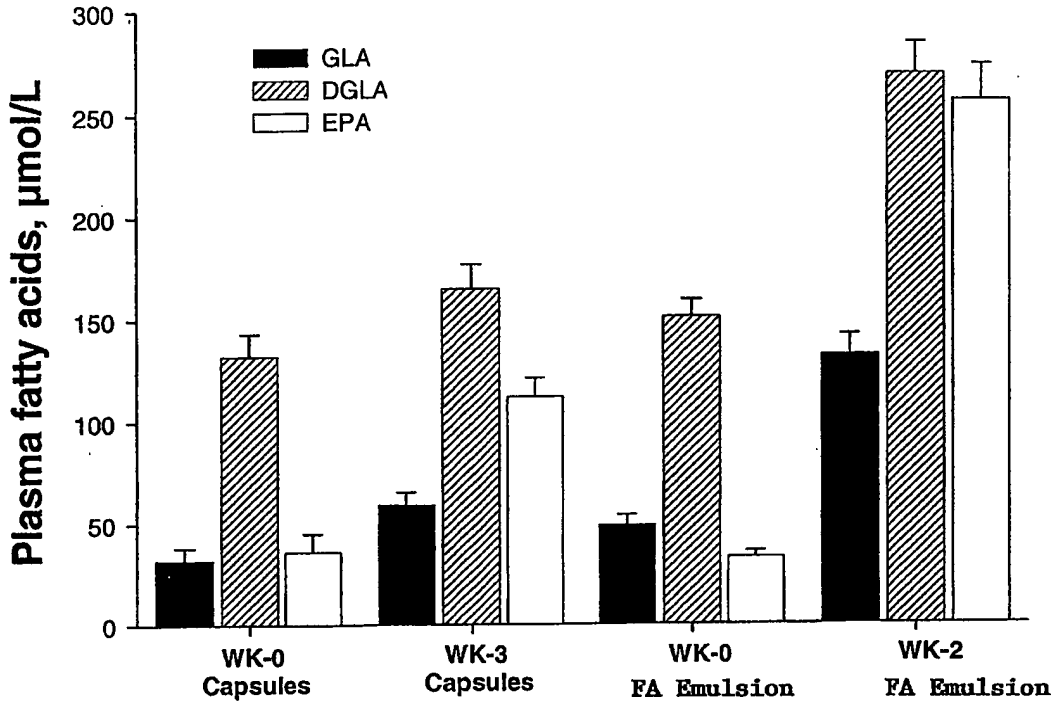

Plasma fatty acids, µmol/L
300
250
200
150
100
50
0
GLA
DGLA
EPA
WK-0 Capsules
WK-3 Capsules
WK-0 FA Emulsion
WK-2 FA Emulsion

FIG. 22A

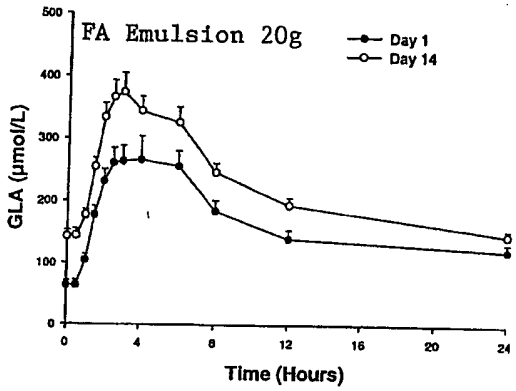


FIG. 22B

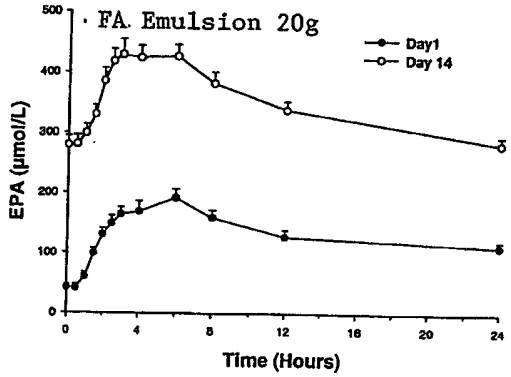


FIG. 22C

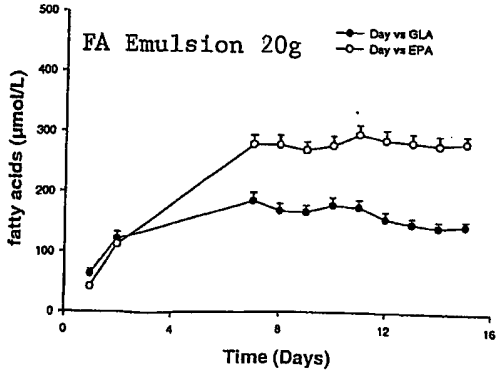


FIG. 23

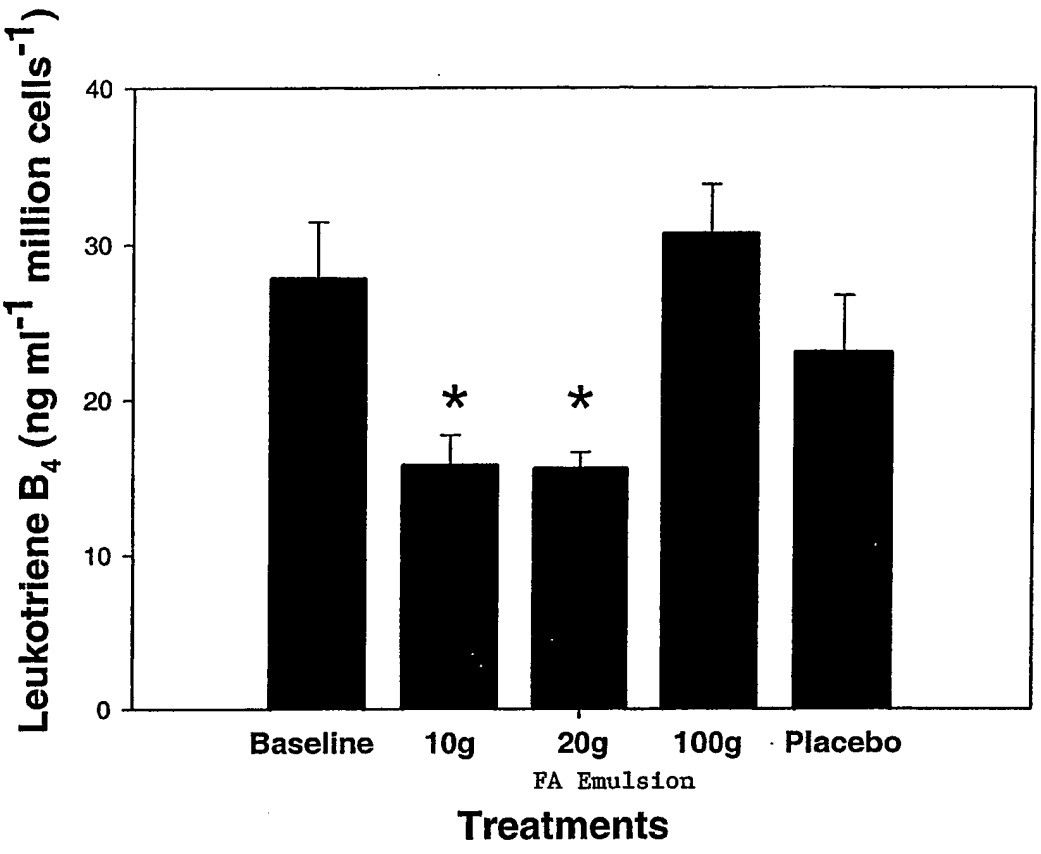

Leukotriene B_4 (ng ml^{-1} million $cells^{-1}$)
40
30
20
10
0
*
*
Baseline
10g
20g
100g
Placebo
FA Emulsion
Treatments

FIG. 24

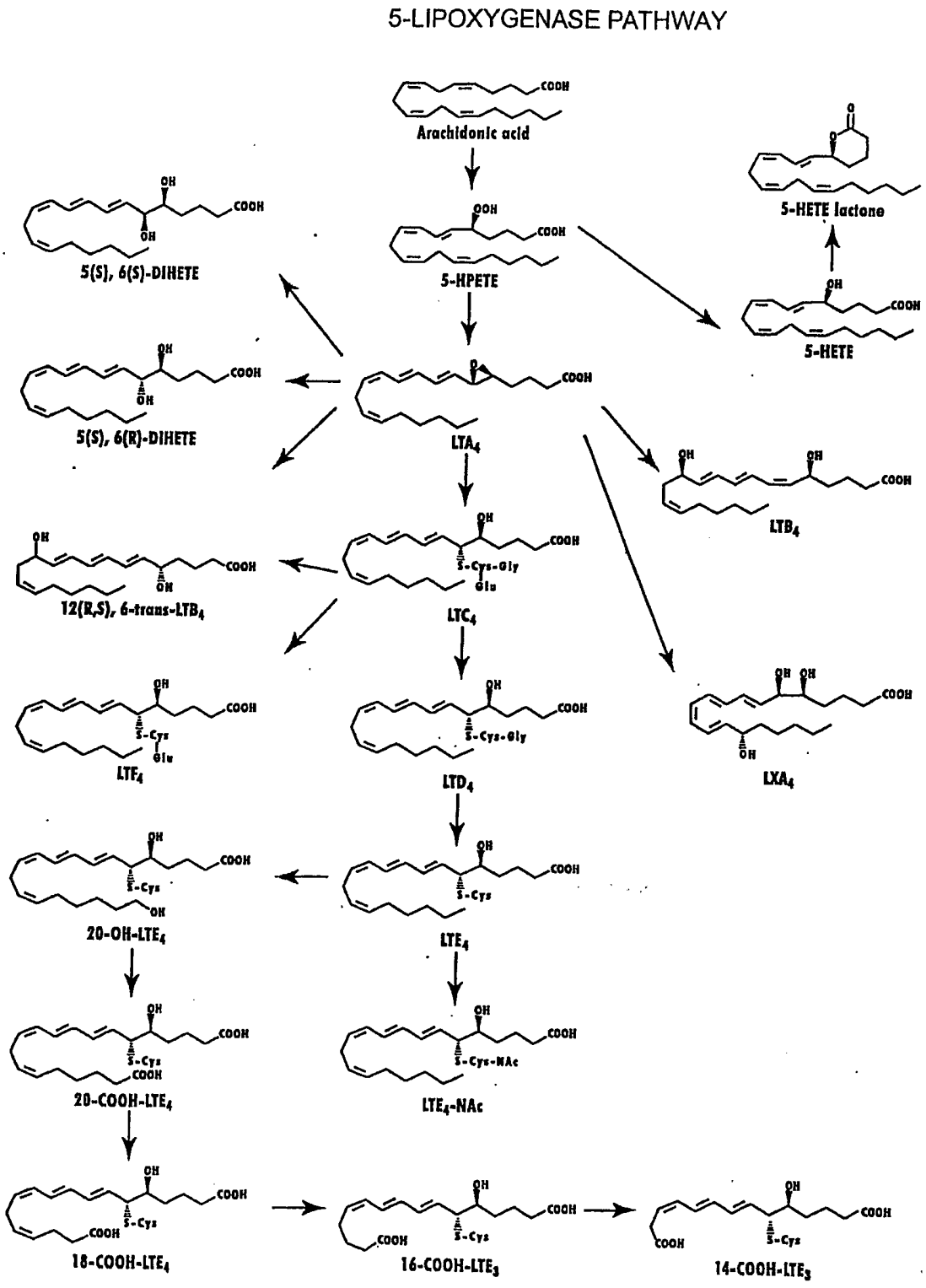

5-LIPOXYGENASE PATHWAY
Arachidonic acid
5-HPETE
5-HETE lactone
5-HETE
5(S), 6(S)-DIHETE
5(S), 6(R)-DIHETE
LTA4
LTB4
12(R,S), 6-trans-LTB4
LTC4
LTF4
LTD4
LXA4
20-OH-LTE4
LTE4
20-COOH-LTE4
LTE4-NAc
18-COOH-LTE4
16-COOH-LTE3
14-COOH-LTE3

FIG. 25

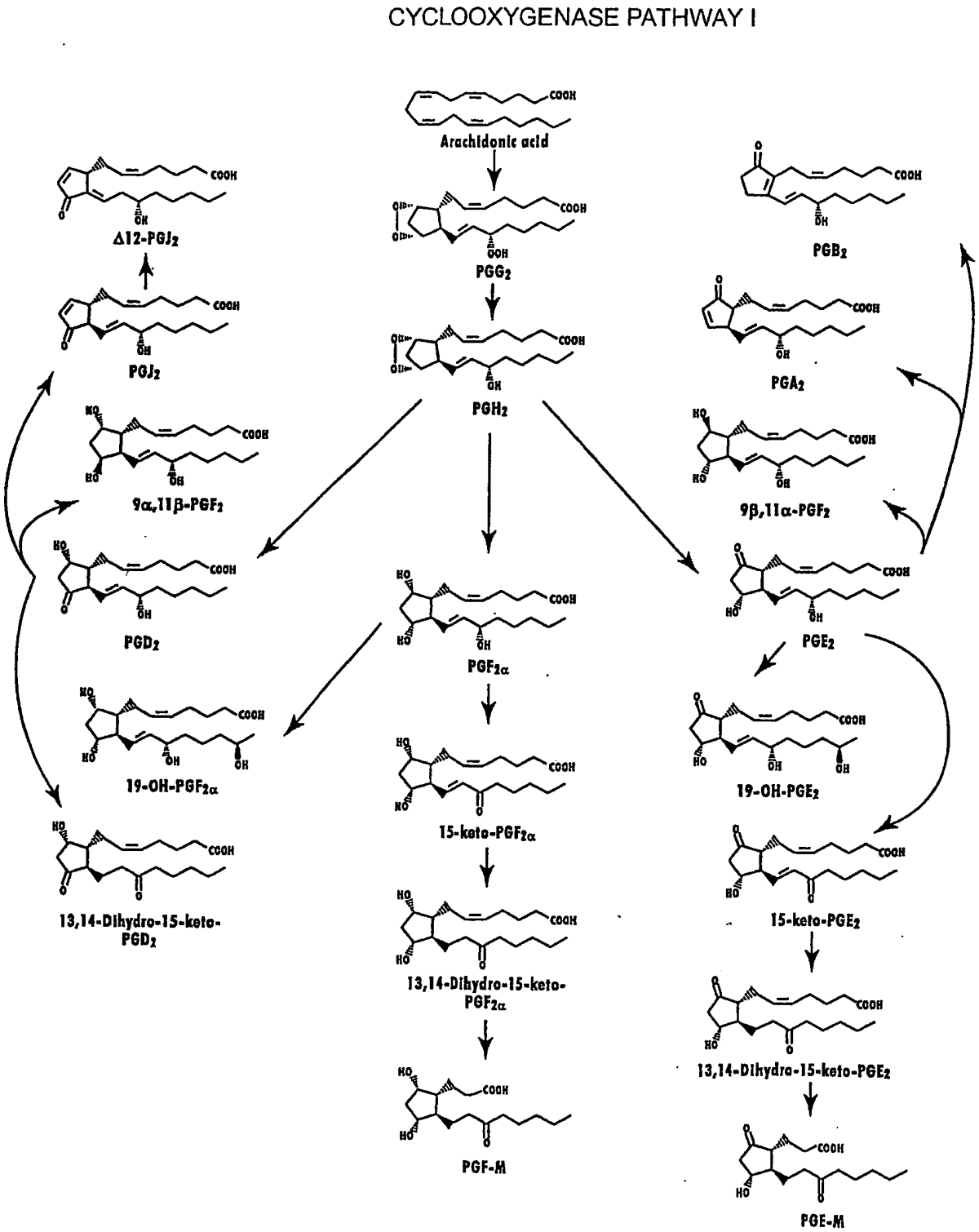
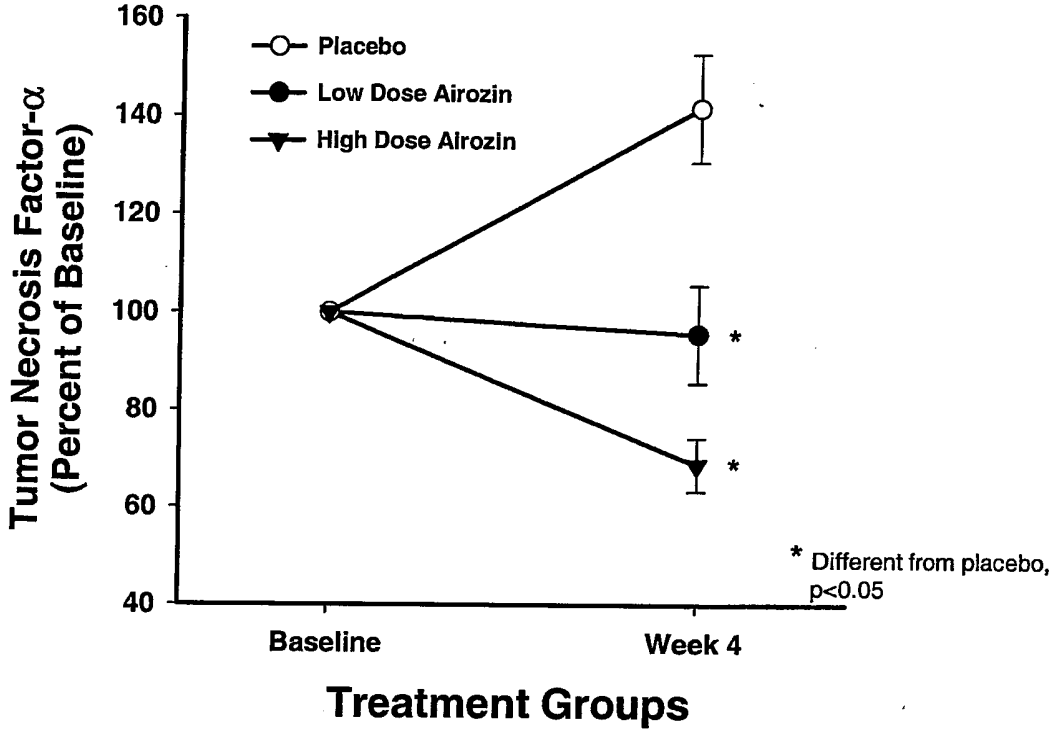

CYCLOOXYGENASE PATHWAY I
Arachidonic acid
PGG2
PGH2
Δ12-PGJ2
PGJ2
9α,11β-PGF2
PGD2
19-OH-PGF2α
13,14-Dihydro-15-keto-PGD2
PGF2α
15-keto-PGF2α
13,14-Dihydro-15-keto-PGF2α
PGF-M
PGB2
PGA2
9β,11α-PGF2
PGE2
19-OH-PGE2
15-keto-PGE2
13,14-Dihydro-15-keto-PGE2
PGE-M

FIG. 26



Low dose group consumed 10g of Airozin per day (0.75g GLA, 0.5gEPA). High dose group consumed 15g Airozin per day (1.12g GLA, 0.75gEPA). Placebo consumed Same formulation as Airozin except Borage and marine oils were replaced by olive oil (no GLA and no EPA).